SOME ASPECTS OF THE AUTECOLOGY OF *APHANIZOMENON FLOS-AQUAE* BORN. ET FLAH. STUDIED UNDER CULTURAL CONDITIONS

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SOME ASPECTS OF THE AUTECOLOGY OF *APHANIZOMENON FLOS-AQUAE* BORN. ET FLAH. STUDIED UNDER CULTURAL CONDITIONS

INTRODUCTION

The algae have been the subjects of cultural investigations since the latter part of the nineteenth century. The main impetus of these researches stemmed from the work of M. W. Beijerinck during the 1890's (60, p.2). Current investigations in the field of algal cultural studies may be in general categorized into the following classification:

1. Physiological investigations using algal cultures as convenient material, especially for studies concerning photosynthesis
2. Cultural investigations relating to morphologic, taxonomic, and life-history studies of algae
3. Cultural work directed at a possible commercial utilization of these organisms *per se*
4. Cultural studies in which the results may be applicable in ecological interpretations

The first category has been of interest to the greatest number of workers, whereas the last-named category has received a negligible amount of attention.

The problem of productivity of the aquatic habitat may be attacked from two directions. One approach may be through ecological investigations on the biotopes and the

other through physiological investigations carried on under controlled conditions. "Neither of these two ways alone, however, leads to the general aim. To reach this, a combination of ecological and physiological methods and data is necessary." (66, p.5)

Pringsheim remarked that the aim in algal cultural work has now to be an integration between laboratory and field work.

> "A still more complete knowledge of taxonomic units is needed for ecological requirements. Otherwise observations on one form will erroneously be extended to similar, but differently adapted forms. In physiology, too, no conformity can be expected between results based on non-identical algal forms, although that has often been done to the great detriment of progress. In the same way experiments sometimes refer to Chlorella indiscriminately . . . results with one of the many different strains of Euglena gracilis have been generalized as covering the whole genus Euglena. It sounds unbelievable, but it is true that almost every strain of Euglena employed in the many physiological investigations bears a wrong name because the authors did not take the trouble to inspect this material under the microscope . . ."

Pringsheim further indicated his belief that most of the physiological investigators confuse rather than elucidate algal taxonomy. This regrettable state of affairs is bound to deteriorate further if ecologists, physiologists, and biochemists continue to use algae for experimental purposes (63, pp.800-803).

Before the work of S. P. Chu (17, pp.285-325) and Wilhelm Rodhe (66, pp.1-149) the planktonic algae had, in general, eluded the cultural habitat. Both of these workers enjoyed considerable success in culturing some of the algal planktonts. Their success did not extend to the culture of cyanophycean species, and to the present time, very few planktonic or nonplanktonic cyanophycean species have been successfully grown in culture. Even those species that have been maintained in culture have proved difficult subjects for well controlled experiments (3, p.34; 14, p.105; 68, p.39).

To date, only G. C. Gerloff and his co-workers have successfully isolated and maintained some of the major blue-green planktonts in culture (e.g. Anacystis cyanea, Gloetrichia echinulata, Aphanizomenon flos-aquae, etc.). Thus far, only one paper has appeared in the literature relating the nutritional requirements of one of these species (31, pp.26-32).

This thesis is a report of cultural investigations of the blue-green planktont Aphanizomenon flos-aquae Born. et Flah. The choice of this organism was based upon a number of considerations. The primary interest stemmed from earlier field investigations and attempts to culture the alga. Aphanizomenon, Anabaena, and Anacystis are the three most notorious cyanophycean bloom

organisms, and Aphanizomenon probably is the most abundant and widespread of these genera occurring in Oregon. In addition, several workers had previously attempted to study this organism under cultural conditions, but none had met with any degree of success.

The initial part of this work was carried on at the University of Wisconsin and was then completed at Oregon State College.

REVIEW OF LITERATURE

A. Historical Considerations

Sachs and Knop, about the 1860's, were the first investigators to grow flowering plants in synthetic cultural media to determine their mineral requirements. Their nutrient solutions consisted of water in which were dissolved the desired inorganic salt or mixture of salts. From these investigations, Sachs and Knop established the major element requirements of higher plants (16, p.48).

Sachs' medium contained the following compounds as nutrients: KNO_3, $Ca_3(PO_4)_2$, $MgSO_4$, $CaSO_4$, NaCl and $FeSO_4$. Knop, recognizing the unessentiality of sodium chloride, simplified his medium to contain the following compounds: $Ca(NO_3)_2$, KNO_3, K_2HPO_4, $MgSO_4$ and $FeSO_4$ (16, p.48). These minerals were supplied in concentrations ranging from 200 mg to 1.0 g per liter of medium (16, p.49).

Famintzin, in 1871, extended Knop's experiments with higher plants to a study of the nutritive requirements of algae. Using Knop's medium, he was apparently the first investigator to successfully grow algae in an inorganic synthetic medium. The work of Famintzen was continued by Molisch (1885-1886) and Benecke (1898) (60, p.1).

Miquel (1890-1892) was the first worker to culture diatoms on an artificial medium, but it was not until

1903 that bacteria-free cultures of these organisms were obtained by Richter (60, p.1). M. W. Beijerinck in 1890 and 1893 claimed to have established algae in pure culture. His experiments involved species of Chlorella and Scenedesmus (3, p.34; 60, p.2; 68, p.25).

The first workers to claim the establishment of pure cultures of blue-greens were Tischutkin in 1897, Bouilhac in 1898, and Beijerinck in 1902. However, E. G. Pringsheim in 1913 established the first unquestionably pure cultures of organisms belonging to this taxon. His pure cultures consisted of two species of Oscillatoria and one of Nostoc (3, p.34). It was not until 1938 that the first unicellular blue-green alga, a species of Chroococcus, was introduced into pure culture (3, p.35).

An important contribution to algal cultural technique was the introduction of agar-agar by Tischutkin in 1897. His cultures were grown in Petri plates containing a layer of agar-agar. This practice still remains popular in contemporary investigations (60, p.2).

Another important advancement in algal-cultural technique was the introduction of soil extract media by Pringsheim in 1912. These media are still successfully used to introduce species into culture that have previously resisted such attempts. Pringsheim suspected

that the efficacy of these media lay in the presence of soluble iron compounds (60, p.6).

Ecological-cultural studies began at an early date. In 1901 Beijerinck made observations on certain blue-green algae (e.g. Nostoc, Anabaena, etc.) and came to suspect these nostocacean forms capable of utilizing atmospheric nitrogen; a conjecture which was later confirmed. He referred to these species as oligonitrophylic organisms (60, p.5).

B. Modern Cultural Procedures

1. General Considerations

In general, algal mineral nutrition is very similar to that of higher plants. A possible exception to this generalization is marked by reports concerning the requirements for calcium (14, p.79; 45, p.57). Very little work has been done on micro-nutrient requirements, which are generally provided for by adding to the culture medium a "shotgun" mixture of minor elements (45, p.57). Not all algal species investigated will grow in an inorganic medium; but by the use of decoctions and biphasic media, it has been possible to introduce a number of species into culture that previously resisted all attempts to be grown on completely synthetic media (60, p.13).

It is, however, only through the use of artificial media of known composition and not from habitat studies that the essential nature of a mineral can be determined (9, p.316). By the use of the following criteria, the essentiality of a mineral is determined:

1. A deficiency of the mineral renders it impossible for a plant to complete the vegetative or reproductive stage of its life history.
2. The deficiency is specific for the element, and it can be corrected or prevented by supplying that element.
3. The element is directly involved in the nutrition of the plant, and is quite apart from possible effects in correcting some unfavorable microbiological or chemical condition of the cultural medium (9, p.322).
4. No other element can be substituted for the essential element (9, p.329).

Contemporary studies of algal physiology utilize growth as a measure of the over-all nutrition or metabolism of the organism (54, p.157).

Until recently, most of the planktonic algal species resisted all attempts to be introduced into inorganic, synthetic media. Chu (17, pp.285-325) in 1942 succeeded in breaking the resistance of a number of these species

to cultural conditions and obtained growth in completely inorganic media. His success seems to have stemmed from imitating natural waters as closely as possible. In this work, the minerals were supplied in very low concentrations as opposed to the copious amounts of nutrients which had previously been used. Chu's results were later substantiated in 1948 by the work of Wilhelm Rodhe (66, pp.1-149).

The last major barrier in planktonic cultural work was broken by G. C. Gerloff et al. (28, pp.216-218; 30, pp.27-44) with the introduction of some of the important planktonic cyanophycean species into culture. Growth of planktonic blue-green species in inorganic media had previously been practically unknown. These investigators utilized one of the synthetic media which had been developed by Chu (Chu number 10 medium).

The only procedure that has enjoyed any degree of success in obtaining pure cultures of blue-green algae has been the employment of ultra-violet irradiation. This method was first introduced by Allison and Morris in their work on Nostoc muscorum (7, p.222), and was later used extensively by Gerloff (30, p.32) and Allen (3, p.41). Fogg reported success in obtaining a bacteria-free culture of Anabaena cylindrica by washing the alga in dilute chlorine water (21, p.79).

2. Media

During the time in which algal cultural studies have been made, many different media have been developed for this purpose. There has been, however, no concurrence concerning the best medium to be used for a particular organism. Furthermore, those media which have been designed for different groups of algae do not vary among themselves more than do the different media which have been designed for the same alga (45, p.58). Most of the algae seem not to be readily affected by small changes in the composition of the medium. If it were otherwise, these species would be readily eliminated in nature. Changes effected in the medium by the metabolism of the occupants, too, are often more extreme than differences among the various media (60, p.33).

In general, there have been two types of media used for culturing algae: (1) those in which natural waters have been enriched, and (2) those which have been completely synthetic and have been composed entirely of purified salts and distilled water (45, p.58). It is only in the latter type that the complete chemical composition of the medium is known. The early media which were employed, and which were listed by Kufferath (47, pp.127-306), are of historical interest only, but this does not necessitate abandoning them (60, p.33).

Those media which were used by Sachs and Knop for culturing flowering plants have been generally too acid for the growth of most algae (60, p.33; 62, p.348). Of the various nutrient solutions that have been used for culturing blue-green algae, certain essential features seem to be common to them all: (1) They comprise weakly alkaline solutions obtained by using K_2HPO_4 or more markedly alkaline solutions obtained by using Na_2SiO_3 or Na_2CO_3; (2) they contain combined nitrogen as calcium or potassium nitrate; and (3) magnesium and iron are included in the formulation (3, p.36).

The most readily available medium, of course, is the natural waters, and these waters were used with considerable success by earlier workers (14, p.76; 60, p.26). Tap water was also employed with considerable success by the earlier investigators. However, the introduction of chlorination and modern plumbing, with its concomitant heavy metal contamination, has made these waters unsuitable for use in cultural media (14, p.76). In the past, distilled water was not suitable for use in media, owing to the toxic effects of copper contamination produced by the early stills (60, p.26). Present workers have been in complete accord in the use of redistilled water, a practice that was introduced by Pringsheim in 1912 (60, p.27). The water

was redistilled in Pyrex or Jena glass stills, or if greater purity was desired, quartz stills were used (60, p.26).

The variable factors in the sundry media have been the source of nitrogen, the concentration of the various minerals, the pH of the medium, the iron content, and special ingredients (e.g. yeast) (62, p.348). Some investigators have used purely inorganic media for the purpose of reducing bacterial growth rather than for strict requirements of the algal population (60, p.32). Recently there has been an attempt to synthesize natural waters by using only inorganic salts in the medium (17, pp.285-325; 66, pp.1-149). The Chu number 10 medium and the Rodhe number VIII medium are quite comparable both in concentration of nutrients in the medium and in composition of the medium. This was not the result of chance, as these media were experimentally developed (66, p.60) and are both physiologically balanced media.

Gerloff et al. (29, p.835) modified the Chu number 10 medium for experimental purposes in working with blue-green planktonts. This modification resulted in a physiologically unbalanced medium in which the essential cations were supplied as chlorides and the essential anions as sodium salts. In this medium, Chu's source of iron ($Fe_2(SO_4)_3$) was replaced by the ferric citrate-

citric acid complex as had been recommended by Rodhe (60, p.43). In addition this medium contained an aliquot of Hoagland's A-Z micro-nutrient solution (29, p.836).

3. Physical Factors

The physical conditions under which algal cultures have been grown have received very little attention in recent investigations (3, p.42). Pringsheim (62, p.348) believed that, as far as possible, natural daylight should be used as a source of illumination, as no artificial illumination had been found which was as good. However, natural daylight was inadequate for critical work (54, p.158), and artificial illumination was provided by means of incandescent lamps until the advent of fluorescent lights (2, p.742; 20, p.580). The main objection to using tungsten lights in cultural work was the large amount of heat dispensed. This problem was usually surmounted by the use of water circulated past the bulbs. Kratz and Myers (46, p.285) in their work with Anacystis nidulans found that equally good growth could be obtained by using tungsten or fluorescent lights as a source of illumination. Algeus (2, p.751) found that fluorescent lights rich in long wave lengths were more satisfactory than those of the daylight type.

The growth of most of the algal species that have been investigated seems to have been favored by low levels of light intensity (100-300 f.c. or 1076-3228 lux). Due to the mutual shading of the cells in the culture flask, the effective light intensity may have been considerably less (3, p.42; 54, p.157). On the other hand, Allen and Arnon (4, p.369) had considerable success in using high light intensities (16,000 lux or ca. 1,480 f.c.) in their experiments with Anabaena cylindrica. In the presence of higher light intensities, it was necessary to maintain the supply of mineral salts and carbon dioxide at a higher level than in cultures radiated at lower light intensities. The earlier workers generally gave their cultures a dark period, but recently continuous illumination of the cultures has been used. The reasons for this change in procedure have not been clear, as very little critical work has been done on the effect of the periodicity of illumination (4, p.369).

Modern investigators have not agreed upon an optimum temperature or temperature range at which their algal cultures ought to be grown. Most investigators have grown their organisms at temperatures around 25° C (3, p.42; 6, p.453; 28, p.216). Chu (17, p.300) found that temperatures of 8-24° C had no effect upon

the growth of his planktonic species. Allison et al. (6, p.435) in their work with Nostoc muscorum found that the vegetative cells of this alga commonly went into a resting condition (not spore formation) at temperatures of 5-10° C, and growth of this species was considerably retarded at 35-40° C. Kratz and Myers, however, (46, p.285) found that the maximum growth of Anacystis nidulans occurred at 41° C. This has been ostensibly the highest optimum temperature recorded for the growth in culture of an alga.

What little information has been available on the effects of aeration with regard to the growth of algae has not been consistent. In some cases culture aeration seemed to be beneficial or even obligatory (14, p.92) while in other experiments this practice seemed to have no effect or to be inimical. Some of the workers aerated cultures by bubbling air through the medium (14, p.92), whereas others achieved aeration by shaking the cultures (2, p.746).

4. Carbon

The normal carbon content of the mineral-free organic fraction of algae has been found to vary from 51-56 percent (45, p.59). Despite the importance of carbon, most of the cultural studies indicated a

general lack of attention for the provision of this element with most of the methods having provided an incommensurate supply (46, p.282). Myers (54, p.158) claimed that growth in cotton-plugged flasks was generally limited by the rate of diffusion of carbon dioxide into the medium.

The species of carbon which are able to enter the algal cell is an unsettled question. It has been generally agreed that the unionized species is able to penetrate into the cell more rapidly than the ionized species (23, p.33). Some investigators have claimed that algae utilized carbon dioxide, some have claimed that bicarbonate was utilized, and some have claimed carbonate was utilized (23, p.33; 45, p.60; 46, p.283; 51, p.269; 54, p.164). Most of these investigators, however, have agreed that their organisms were unable to directly utilize the carbonate ion (23, p.35). The proportion of carbon dioxide, bicarbonate, and carbonate which exists in the medium is a function of the pH, the temperature, and the excess base. There has been an assumption that bicarbonate ions act indirectly in supplying undissociated carbon dioxide (23, p.34; 45, p.60). In addition, Fogg (23, p.34) claimed that bicarbonate ions may be absorbed directly by active mechanisms.

Some of the organisms have been dependent upon a supply of carbon, as carbon dioxide, entering the media by diffusion (54, p.158), some have been supplied with carbon in the form of carbonate (46, p.283), and some have been provided carbon by means of bubbling air or 3-5 percent carbon dioxide in air or nitrogen through the medium (45, p.60; 46, p.282; 54, p.158).

At the present time, the carbon question has not been settled and is in need of further exploration (54, p.164).

5. Nitrogen

Nitrogen has been considered to be one of the important limiting factors in the development of phytoplankton (66, p.92). As early as 1889, Frank reported that some of the algae were able to utilize atmospheric nitrogen. This report came four years before the first isolation of a nitrogen-fixing bacterium was announced (23, p.68). It was not, however, until 1928 that this phenomenon was definitely established in blue-green algae by Drewes (13, p.264). Nitrogen fixation is not a catholic phenomenon among the blue-green algae (75, p.304; 23, p.69) and is apparently restricted to some species within the Nostochineae. Progress in studying nitrogen fixation among the

blue-green algae has been considerably retarded by the inability to secure bacteria-free cultures (7, p.222). Nitrogen fixation has also been reported to be suppressed by the presence of combined nitrogen in the medium (54, p.162). Attempts to demonstrate nitrogen fixation in other divisions of the algae have been unsuccessful (14, p.79; 23, p.69), and this phenomenon seems to be restricted to myxophycean species.

The nitrogen content of algae has been found to be comparable to that found in other micro-organisms (7-11 percent of the dry weight), and is considerably above that of most higher plants (54, p.161). There has been a general agreement that algae absorb and utilize nitrogen in the combined form either as ammonium or nitrate (3, p.45; 45, p.62; 60, p.36; 66, p.53). Pringsheim (60, p.36) stated that no alga was known that could not utilize either form of nitrogen, but Fogg (23, p.71) remarked that some flagellates were only able to utilize ammonium nitrogen during chemotrophic growth. Nitrite utilization has been almost totally neglected in algal nutritional studies. This form of nitrogen is relatively unimportant in the natural habitat, and some cases have been known in which it induced a toxic effect upon the growth of organisms in culture (60, p.35). Fogg and Wolfe (25, p.107) found that at 27 ppm, nitrite

completely inhibited the growth of Anabaena cylindrica. In impure cultures, nitrite may be oxidized to nitrate (14, p.77).

Before being incorporated into cellular constituents, nitrates are reduced to ammonia (60, p.36), and investigations have indicated that if both nitrate and ammonium were present in the medium, the ammonium was absorbed in preference to the nitrate (31, p.31; 54, p.162; 56, p.333; 60, p.36). The absorption of ammonium was paralleled by a lowering of the pH of the medium, whereas the absorption of nitrate was accompanied by an increase of the pH (23, p.75; 45, p.62; 66, p.53). As the lower pH's are generally deleterious to the growth of most algal species, nitrogen in the medium has usually been supplied in the form of nitrate (6, p.444; 23, p.75).

Various workers have supplied a wide range of nitrogen concentrations in the medium. Some species have proved to be ostensibly insensitive to variation in the nitrogen concentration (e.g. Chlorella) as long as it has not been limiting (45, p.63), whereas the growth of some species was inhibited at relatively high concentrations (e.g. Nitzschia at 70 ppm). In some species, inhibition was initiated at relatively low

concentrations (e.g. Pediastrum at 8.7 ppm) (17, p.301; 18, p.128; 45, p.63). Chu's experiments (18, p.128) indicated there was an optimum range of nitrogen concentration for each of the planktonts he studied. The upper optimum concentration in these studies varied from 6.5 to 17.0 ppm of nitrogen. Gerloff's work with blue-green planktonts (29, p.837; 31, p.27) indicated an optimum nitrogen concentration of 13.6 ppm; this was the nutrient required in the largest quantity.

6. Phosphorus

Phosphorus has also been credited with exerting a limiting effect upon the growth of phytoplanktonts in nature (66, p.64). In natural waters orthophosphate has been the form normally found, and it is this form that has generally been supplied in the various media (45, p.66). Pyrophosphate has been found to be not as readily utilizable as orthophosphate (13, p.263).

Before the work of Chu, the phosphorus concentration in the sundry media varied from 9-110 ppm, and this investigator was apparently the first to study the growth of phytoplankton as it was affected by the concentration of phosphorus (18, p.142). Chu's work indicated that the phosphorus concentration normally found in natural waters (0.003-0.02 ppm) was not sufficient to induce inhibition

of growth of his phytoplanktonic species. In all of the organisms he studied, the inhibitory effects of phosphorus occurred at or above 17.9 ppm (18, p.143). However, the optimum phosphorus requirements of phytoplanktonic species that have been studied seem to be above the concentrations occurring in natural waters (66, p.64).

The results of Rodhe's experiments (66, pp.89-90) indicated that various planktonts have specific levels of phosphorus requirements, and it is incorrect, according to Rodhe, to regard phosphorus as a limiting growth factor in all planktonic algae.

Gerloff (29, p.840; 31, p.27) found that the growth of Coccochloris and Anacystis decreased when the concentration of phosphorus fell below 0.45 ppm and 0.18 ppm respectively. In these experiments the nitrogen/phosphorus ratio for optimum growth was 30/1 for Coccochloris and 75/1 for Anacystis. Blinks in his work (13, p.263) found that when ammonium was used as a source of nitrogen, there was a lower requirement for phosphorus than when nitrate was used as a source of nitrogen.

7. Iron

Iron, as well as nitrogen and phosphorus, has often been considered to be a limiting factor in the development of phytoplankton (66, p.104). At the present time a

requirement for iron has been well substantiated, but the manner in which it should be provided has been controversial (45, p.69).

Many of the older formulations did not specify the addition of iron to the medium, but it was probably introduced as an impurity in the other ingredients (14, p.80). In those media to which iron was specifically provided, it was commonly added as a chloride or sulphate (35, pp.366-367). In alkaline solutions the concentration of soluble iron is very low as it is oxidized to the ferric form and precipitated (45, p.69). Hopkins and Wann (35, p.366) added sodium citrate to their culture solutions. With the addition of this compound, they were able to maintain a greater amount of iron in solution. They also found that part of the iron was lost from solution by becoming adsorbed on precipitates of calcium phosphate (35, p.367). In a later work (36, p.421) these same investigators ascertained that the concentration of iron necessary for good growth was considerably reduced with the addition of sodium citrate to their medium. Previous results without the addition of the citrate compound required a larger iron concentration in the medium. These workers, however, obtained negative results with the addition of potassium and sodium tartrate to the medium inasmuch as the availability

of iron was not seemingly increased. Pringsheim's success with soil extract media has often been attributed to the maintenance of iron in a soluble form (60, p.6). The same generally good results have been obtained with the addition of natural humic acid or synthetic iron humate to culture media (6, p.440).

Rodhe (66, p.35) found that iron added as ferric citrate was unstable in the following manner:

Ferric ions + citrate ions + water $\longrightarrow$

Ferric hydroxide + citric acid

In further prosecuting this research, he ascertained that by adding an equal amount of citric acid with the ferric citrate he was able to maintain an ascensional amount of reactive iron in solution. This procedure was later utilized by Gerloff et al. (28, p.216) in their modification of the Chu number 10 medium. The use of ferric chloride as an iron source by Chu has often been a source of criticism of his work. Gerloff's work with Coccochloris indicated that at higher concentrations (above 1.0 ppm) equally good results could be obtained in the growth of this organism by the use of ferric citrate-citric acid or by using ferric chloride. However, in lower concentrations, when iron was added as ferric citrate-citric acid, the resultant growth was superior to that achieved with iron supplied as

ferric chloride (29, p.839). Due to the uncontrollability of iron in his alkaline medium, Holm-Hansen (37, p.21) placed no significance upon his growth results as related to various concentrations of iron.

8. Calcium

Calcium has been the only nutrient element for which algal requirements have been reported to be at variance with those of vascular plants (54, p.161). Some of the green algae and one blue-green planktont (Coccochloris) have been found to require no calcium in their nutrition (29, p.838; 45, p.67; 66, p.54).

Allison et al. in their work with Nostoc muscorum (6, p.454) found that this alga required neither calcium nor strontium for its growth. Their results did indicate, however, that calcium was necessary for growth in this alga when it utilized atmospheric nitrogen.

Many of those experiments which disclaimed the necessity for calcium in algal nutrition involved the use of chemicals which had not been previously purified, and calcium in sufficient quantities for normal growth may have been introduced as a contaminant. It still remains, though, that if calcium was required in the metabolism of these organisms, it was in very small quantities and approached concentrations approximating

those necessary for trace elements. The results of Allen and Arnon (4, p.368) in working with Anabaena cylindrica were seemingly diametrical to other investigations. These investigators found that their alga required at least 20.0 ppm of calcium for optimum growth, thereby resembling the higher plants in nutrition. These results, too, revealed that metabolically calcium was not replaceable by strontium. Contrarily, the work of Holm-Hansen showed that calcium above a concentration of 21.0 ppm inhibited the growth of Calothrix. He found, however, there was a definite requirement for this element at lower concentrations. He concluded that the detrimental effects resulted from an antagonism of calcium and other ions (37, p.18). At higher concentrations there may also have been a precipitation of phosphorus and iron (60, p.38).

9. Potassium and Sodium

Potassium has been considered to be necessary in the development of phytoplankton, but investigators have generally agreed that it is not a limiting factor in the natural habitat (66, p.118).

The evidence for a need of potassium and/or sodium in cultural media has been unclear. Ketchum (45, pp.68-69) remarked that no evidence existed for a sodium requirement

in algal nutrition, but that potassium was definitely necessary for growth. Gerloff (29, p.837) found that in the nutrition of Coccochloris potassium was the cation required in the largest quantity.

Emerson and Lewis (20, p.580), on the other hand, found that their cultures of Chroococcus failed to grow if sodium were omitted from the medium. These same workers were also able to substitute sodium to a large extent for potassium, but poor growth resulted if potassium were completely omitted from the medium. Kratz and Myers (46, pp.283-284) found that Anabaena variabilis and Anacystis nidulans required both sodium and potassium. In this work they found it possible to vary greatly the potassium/sodium ratio without affecting the growth of the alga.

Allen (3, p.45) and Allen and Arnon (5, pp.653-655) also found sodium to be necessary in the growth of their blue-green organisms. In the growth of Chlorella and Ankistrodesmus, Myers (54, p.159) indicated that potassium could be partially replaced by rubidium, but not by sodium or cesium.

10. Sulphur

Sulphur has been generally added to the medium and taken up by algae in the form of sulphate (45, p.67).

Gerloff (29, p.838; 31, p.28) found that sulphur was required in larger quantities than phosphorus, and next to nitrate, sulphate was the anion demanded in the largest quantity. No studies on the variation of sulphur concentration as related to growth have been reported in the literature.

11. Magnesium

Magnesium is conceded to be necessary for the growth of phytoplankton (45, p.67), but it is not generally limiting in nature. The requirements of most species under cultural conditions seem to have been quite low (0.1-1.0 ppm) (31, p.27; 45, p.68).

12. Silicon

Silicon has been shown not to be essential in the nutrition of algae other than diatoms (45, p.70). Sodium silicate added to cultural media apparently benefited the growth of many forms other than diatoms, but this was probably due to the weakly alkaline reaction of this compound (66, p.54).

13. Trace Elements

With the production of reagent chemicals of greater and greater purity, the older media came to

provide insufficient support for good growth. This situation arose from a deficiency of certain essential elements present as contaminants in the older reagents (42, p.163). Some of the success obtained by using soil extract media may also have been due, in part, to the provision of minor elements (60, p.40).

In contemporary investigations, the trace elements have usually been supplied in a "shotgun" fashion to the medium as Hoagland's A-Z solution (34, p.291) or as Arnon's modification of this solution (8, pp.322-325). At the present time, zinc, boron, copper, molybdenum, and cobalt have been determined to be necessary in the growth of some species (12, p.539; 24, p.434; 25, pp.112-113; 43, p.554; 45, p.70; 69, p.40). In cyanophycean organisms, the only minor elements that have been definitely established as being necessary are cobalt and molybdenum which have been related to nitrogen fixation (3, p.46; 38, p.667).

14. Hydrogen-ion Concentration

The effect of pH *per se* on growth has proved very difficult to study and has been very poorly understood (11, p.525). Buffers added to the medium at concentrations necessary to prevent pH changes have usually been

toxic or metabolically active (60, p.38; 62, p.348; 73, p.520).

The pH of the medium is generally altered by the activities of the organisms, and these shifts in hydrogen-ion concentration may be responsible for secondary deleterious effects (e.g. precipitation of calcium, magnesium, and iron) (14, p.94; 35, p.360). The largest changes in the pH of the medium have been associated with nitrogen absorption. When nitrogen was supplied in the form of ammonium, the pH dropped as this substance was absorbed. Conversely, if the source of nitrogen used were a nitrate, the pH of the medium increased as this ion was removed from the medium.

General concensus has been that cyanophycean algae required a slightly alkaline medium for growth (3, p.47; 14, p.93; 46, p.285). Gerloff found that maximum growth of Coccochloris occurred between pH 10.5 and 11.0, whereas the maximum growth of Anacystis occurred at pH 10.0 (29, p.838; 31, p.30). Allen (3, p.47) found the optimum pH for growth of a species of Oscillatoria was between pH 10.0-11.0. In addition, she found that several other species of blue-greens were able to grow well at these high pH levels. Bacterial growth, however, has been found generally to be limited to pH levels below 8.0 (72, p.143). In media designed for blue-green

algae, an alkaline reaction has been usually effected by the addition of either sodium silicate or sodium carbonate (28, p.217).

C. Aphanizomenon

Contemporary taxonomy has recognized a number of species belonging to the genus Aphanizomenon (26, pp.824-827; 39, pp.191-193). In this country only three of the species have been known to occur (58, pp.1-85; 65, pp.326-329; 71, p.586). Two of these species, A. americanum Reinh. and A. occidentalis Phinney and McLachlan have been found to be limited in distribution and have been known only from the United States.

One species, A. flos-aquae Born. et Flah. has been found to be widely distributed both on this continent and abroad and was one of the most common and abundant blue-green planktonts found in Oregon (58, pp.1-85). This species has often been a major component of bloom populations and has been in part responsible for the foul "pigpen" odor associated with these conditions (19, p.118; 55, p.111, 67, p.129). These blooms generally occurred during the warm summer months, but A. flos-aquae has also been known to bloom under the

ice (50, p.129; 59, p.528). It has been generally during the colder months that the overwintering akinetes were formed (39, p.192; 67, p.31; 70, p.62).

Responsibility for some toxic conditions has been delegated to A. flos-aquae. Cases of fish and stock poisoning have been reported, but no human intoxications have been directly related to, or specifically reported for, Aphanizomenon (44, pp.80-84; 69, p.54).

Attempts to introduce this alga into culture have, on the whole, disappointed those who have tried. The first recorded attempt was made by E. T. Rose (67, pp.129-141). Germination of akinetes occurred in Rose's cultures, but he was unable to purify the cultures or to maintain them for any length of time. Rodhe (66, p.61) tried many different media including decoctions, lake water, and sundry physical conditions in attempting to maintain the alga in culture. These cultures could be maintained for a short period, but there was always a cessation of growth. Gerloff et al. (30, p.28) were successful in isolating and maintaining this organism in culture. These workers were able to carry the organism through a program of continuous subculturing, but were unable to carry on experimental work due to erratic growth.[1] It was possible, however,

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[1]Personal communication with Dr. G. C. Gerloff.

to determine that this alga was not able to fix free nitrogen (75, p.340).

Holm-Hansen (37, p.73) also attempted to perform experimental work with Aphanizomenon, but without success.

> "In all experiments with Calothrix, Nostoc, Coccochloris and Microcystis growth has been remarkably uniform and consistent within each treatment. Not a single of any of the above species has failed to support good growth when the medium was complete. In sharp contrast to this has been the behavior of Aphanizomenon flos-aquae. Its growth has been extremely inconsistent and unpredictable in most experiments. In some experiments, some or all of the cultures failed to grow; even within treatments where one would expect rather similar results, growth has often varied from little or none to very good. The following factors have been studied and have failed to provide an explanation for the lack of consistent growth by Aphanizomenon: Concentration of micro-elements, size of inoculum, total concentration of salts, light intensity, light duration, effect of shaking cultures 3-4 times daily, relative concentrations of the major elements, and the supplying of extracts of mold, yeast, and malt. The most promising of these was the addition of leaf-mold extract prepared as described by Pringsheim. In many experiments this had a very beneficial effect on growth, but even in this case the lack of consistency was too great to reach definite conclusions."

MATERIALS AND METHODS

A. Organisms Used

Two strains of Aphanizomenon flos-aquae Born. et Flah. were used in this investigation. These cultures were obtained from, and had been isolated by, Dr. G. C. Gerloff of the University of Wisconsin. After these organisms had been introduced into culture, they were identified by Dr. Francis Drouet of the Chicago Museum of Natural History. The culture referral numbers in the University of Wisconsin algal culture collection are 1032 and 1062. These same numbers appear on the specimens filed in the Cryptogamic Herbarium of the Chicago Natural History Museum. These cultures of Aphanizomenon were originally obtained from plankton Collections from lakes in southeastern Wisconsin. By repeated subculturing, unialgal cultures were obtained.

The number 1032 strain of Aphanizomenon was originally isolated in 1948 in a slightly modified form of the number 10 medium of S. P. Chu (Table 1). The only modification consisted of replacing ferric chloride by a ferric citrate-citric acid mixture. The number 1062 strain of Aphanizomenon was isolated at a later date in Gerloff's modification of Chu's number 10 medium (Table 2). Stocks

of 1032 and 1062 were carried on the Gerloff number 10 medium (Table 2).

Strain 1032 was reported in the literature to be unialgal and to have been rendered bacteria-free by ultra-violet irradiation (30, p.28). Later this culture became contaminated, or perhaps the bacterial population had only been greatly suppressed by the initial ultra-violet treatment, and the culture was again irradiated with ultra-violet light. The bacteria were again ostensibly eliminated, but contamination appeared a second time. In addition to ultra-violet treatments, this culture had been subjected to treatments with a number of antibiotics. The results of these experiments proved to be unsuccessful in eliminating the bacteria. As reported in the present investigation, strain 1032 of Aphanizomenon was not in a bacteria-free condition, and no attempt was made by the writer to render it so.

As indicated previously, strain 1062 was isolated at a later date, and its presence has never been announced in the literature. No attempt was made either by Dr. Gerloff or the writer to eliminate the bacteria from this culture. As reported here, this culture, although unialgal, was contaminated with bacteria.

As it occurs in nature, the trichomes of A. flos-aquae adhere laterally, forming spindle-shaped, flake-like

colonies in which the filaments are of a determinant length. The trichomes are attenuated at their apices, and the terminal cells are generally unpigmented. Cylindrical heterocysts are present at an intercalary position, one within each trichome. Generally, toward the end of the growing season, cylindrical akinetes are present. These spores are also found in a median intercalary position but not adjacent to the heterocysts; rarely more than one spore arises in each trichome.

Under cultural conditions, strain 1032 did not form colonies characteristic of the species. The filaments in culture were of an indefinite length, and the filaments grew in a greatly entangled mat. In a healthy condition this alga was of a bright, dark green color. Heterocysts were common within the filaments, but akinetes were never observed. Strain 1062, as it was grown under cultural conditions, formed the characteristic flakes of the species; the flakes, however, deliquesced at their margins. In a healthy condition this strain was of a paler green color than 1032. As in the case of 1032, heterocysts were present, but akinetes were not known to occur.

B. Preparation of the Media

During the course of this investigation, only Pyrex glassware was used. The glassware was cleaned in a

solution consisting of potassium dichromate and concentrated sulphuric acid in which it was submerged and allowed to steep for at least one-half hour. It was then removed from the acid solution, rinsed in copious amounts of tap water, and finally rinsed at least seven times in distilled water and allowed to dry.

All of the nutrient compounds used in this investigation were prepared as stock solutions of a convenient concentration for dilution to the final concentration used in the medium. These stock solutions were prepared by dissolving analytical reagent grade compounds in the requisite amounts of glass-distilled water using volumetric glassware. The prepared stock solutions were stored in glass-stoppered Pyrex bottles from which aliquots of the proper amount were removed with volumetric pipettes in formulizing the final dilutions, again using glass-distilled water.

C. Media Used

The original isolate of strain 1032 was obtained using a physiologically balanced medium, the number 10 medium developed by S. P. Chu (17, p.299). This medium will hereafter be referred to as the C-10 medium (Table 1).

Gerloff's initial investigations led to the development of a physiologically unbalanced medium. Concentrations of

Table 1

Chu No. 10 Medium*

Compound	Grams/liter culture medium
$Ca(NO_3)_2$	0.0400
K_2HPO_4	0.0100
$MgSO_4 \cdot 7H_2O$	0.0250
$NaCO_3$	0.0200
Na_2SiO_3	0.0250
$FeCl_3$	0.0008

*Chu, S. P. (17, p.298)

In the Chu medium first used by Gerloff, the ferric chloride was replaced by the ferric citrate-citric acid complex; 0.0030 grams/liter of each of these two compounds were used.

the essential elements used in this medium were the same as had been used in the C-10 medium. In this medium, the essential anions were supplied as salts of sodium and the essential cations as chlorides (Table 2). All of the cultures at the University of Wisconsin were carried on this medium. Subsequent investigations by Gerloff, working with Coccochloris and Anacystis, indicated that this medium was deficient in nitrogen. The nitrogen concentration was thereupon increased threefold to 20.4 ppm; other minor modifications were also introduced. In using this medium, Gerloff added 1/25 the concentration of Arnon's micro-nutrient solution (Table 3) that had been recommended for higher plants (8, pp.322-325). This is a modification of Hoagland's A-Z micro-nutrient solution (34, p.291). Stock cultures of both Anacystis and Aphanizomenon were eventually carried on this medium. This medium will hereafter be referred to as the G-10 medium (Table 4). Later the writer slightly modified the G-10 medium by introducing minute variations in the concentration of certain ions to allow a greater latitude of adaptability and ease in preparing the medium and its variations. This medium will also be referred to as G-10 (Table 5).

The results of the present investigation with Aphanizomenon led to a modification of the G-10 medium. In the preparation of this medium, G-10 stock solutions

Table 2

Gerloff's Modification of the Chu No. 10 Medium

Element	mg of element/l	Compound	mg of compound/l	mg/ml in stock solution	ml of stock solution/l of medium
nitrogen	6.8	$NaNO_3$	41.3	4.13	10.0
sulphur	3.3	Na_2SO_4	14.6	1.46	10.0
phosphorus	1.8	$Na_2HPO_4 \cdot H_2O$	8.2	0.82	10.0
potassium	4.5	KCl	8.6	0.86	10.0
magnesium	2.5	$MgCl_2 \cdot 6H_2O$	20.9	2.09	10.0
calcium	9.8	$CaCl_2 \cdot 2H_2O$	35.9	3.59	10.0
carbonate	22.7	Na_2CO_3	40.0	4.00	10.0
silicon	5.7	$Na_2SiO_3 \cdot 9H_2O$	58.2	5.82	10.0
iron	0.56	ferric citrate citric acid	3.0 3.0	0.30 0.30	10.0

The ferric citrate and citric acid were mixed together and 10 cc of the mixture was pipetted. This solution was kept in the refrigerator covered with a paper sack. A fresh stock solution should be prepared at least once every two weeks.

Table 3

Composition of Arnon's Micronutrient Solution*

This solution is made up in three portions: A-4, B-7, and C-13. This solution was designed for use with higher plants. In the experiments on Aphanizomenon flos-aquae, 1/25 the concentration recommended for higher plants was used.

A-4 Solution

Using one cc of this solution per liter, the following concentrations are obtained in parts per million:

boron	0.50
manganese	0.50
zinc	0.05
copper	0.02

Preparation of Solution

Compound	Grams per liter
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.18
$ZnSO_4 \cdot 7H_2O$	0.222
$CuSO_4 \cdot 5H_2O$	0.079

*Arnon, D. I. (8, pp.322-325)

Table 3 (Cont.)

B-7 Solution

To prevent precipitation, all constituents are dissolved in N/10 sulphuric acid.

Using one cc per liter, 0.01 ppm is delivered for each of the following: molybdenum, vanadium, chromium, nickel, cobalt, tungsten, and titanium.

This solution contains the following compounds in milligrams per 10 liters of N/10 sulphuric acid.

Compound	mg/10 l
MoO_3 (85%)	176.4
NH_4VO_3	229.6
$Cr_2K_2(SO_4)_4 \cdot 24H_2O$	960.2
$NiSO_4 \cdot 6H_2O$	447.8
$Co(NO_3)_2 \cdot 6H_2O$	493.8
$Na_2WO_4 \cdot 2H_2O$	179.4

The titanium is prepared by dissolving 736.6 mg of $TiO:(COO \cdot COOK)_2 \cdot 2H_2O$ in water, precipitating the titanium with ammonium hydroxide, filtering, dissolving the precipitate in N/10 sulphuric acid and combining with the rest of the solution.

Table 3 (Cont.)

C-13 Solution

Using one cc per liter, a concentration of 0.005 ppm is delivered for each of the following elements: aluminum, arsenic, cadmium, strontium, mercury, lead, lithium, rubidium, bromine, iodine, fluorine, selinium, and beryllium.

This solution contains the following compounds in milligrams per 10 liters of water.

Compound	mg/10 l
$Al_2(SO_4)_3$	317.1
As_2O_3	66.1
$CdCl_2$	81.5
$SrSO_4$	104.9
$HgCl_2$	67.7
$PbCl_2$	67.1
LiCl	305.5
Rb_2SO_4	78.1
NaBr	64.4
KI	65.4
NaF	110.5
Na_2SeO_4	119.4
$Be(NO_3)_2 \cdot 3H_2O$	1,037.0

The A-4, B-7, and C-13 solutions are appropriately diluted with glass-distilled water and then combined into one solution for use. One cc/liter of medium is used.

Due to the unavailability of sodium selenate, selenic acid was substituted (H_2SeO_4-sp. gr. 1.4). This compound was used by Hoagland in the preparation of his A-Z solution (34, pp.288-294).

Table 4

Gerloff No. 10 (G-10) Culture Solution

Element	mg of element/l	Compound	mg of compound/l	mg/ml in stock solution	ml of stock solution/l of medium
nitrogen	20.4	$NaNO_3$	123.9	4.13	30.0
sulphur	1.65	Na_2SO_4	7.3	1.46	5.0
phosphorus	0.9	$Na_2HPO_4 \cdot H_2O$	4.1	0.82	5.0
potassium	2.25	KCl	4.3	0.86	5.0
magnesium	0.5	$MgCl_2 \cdot 6H_2O$	4.2	2.09	2.0
calcium	0.98	$CaCl_2 \cdot 2H_2O$	3.6	3.59	1.0
carbonate	22.7	Na_2CO_3	40.0	4.00	10.0
silicon	5.7	$Na_2SiO_3 \cdot 9H_2O$	58.2	5.82	10.0
iron	0.56	ferric citrate citric acid	3.0 3.0	0.30 0.30	10.0
minor element solution	-	-	-	-	1.0

Table 5

Modified Gerloff No. 10 Culture Medium for Ease of Variation

Element	mg of element/l	Compound	mg of compound/l	mg/ml in stock solution	ml of stock solution/l of medium
nitrogen	10.0	$NaNO_3$	60.7	6.07	10.0
sulphur	2.0	Na_2SO_4	8.9	0.89	10.0
phosphorus	1.0	$NaH_2PO_4 \cdot H_2O$	4.5	0.45	10.0
potassium	1.0	KCl	1.9	0.19	10.0
magnesium	1.0	$MgCl_2 \cdot 6H_2O$	8.5	0.85	10.0
calcium	2.0	$CaCl_2 \cdot 2H_2O$	7.3	0.73	10.0
carbonate	22.7	Na_2CO_3	40.0	4.00	10.0
silicon	5.0	$Na_2SiO_3 \cdot 9H_2O$	51.0	5.10	10.0
iron	1.0	ferric citrate citric acid	5.3 5.3	0.53 0.53	10.0
minor element solution	-	-	-	-	1.0

were used, but the concentration of the nutrients differed. This medium will be referred to as the M-O medium (Table 6).

Some of the investigations reported here utilized the physiologically balanced medium developed by Wilhelm Rodhe (66, p.57) which will be referred to as the R-VIII medium (Table 7). In the preparation of the R-VIII medium, the stock solution of sodium silicate prepared for use in the G-10 medium was used as a source of silicon. This eliminated the necessity of preparing two stock solutions of sodium silicate.

A summary of the concentration of elements used in the various media is presented in Table 8.

D. Method of Making Transfers

In handling and growing the alga, aseptic conditions were observed throughout, unless the experimental conditions dictated otherwise. After the medium had been placed in flasks, it was autoclaved at 15 pounds pressure for 20 minutes. The flasks were then taken from the autoclave and allowed to stand for at least 24 hours before they were inoculated. At the University of Wisconsin, the flasks were allowed to stand in the laboratory, as there was only a slight differential in temperature between the culture room and the laboratory. At Oregon State College, the flasks were placed in the culture room

Table 6

Preparation of the G-10 and M-0 Culture Media

Element	G-10 (Modified)			M-0		
	mg of element/l	mg of compound/l	ml of stock solution/l of medium	mg of element/l	mg of compound/l	ml of stock solution/l of medium
nitrogen	20.0	121.4	20.0	15.0	91.1	15.0
sulphur	1.0	4.4	5.0	2.0	8.9	10.0
phosphorus	1.0	4.5	10.0	1.0	4.5	10.0
potassium	2.0	3.8	20.0	2.0	3.8	20.0
magnesium	0.5	4.3	5.0	1.0	8.5	10.0
calcium	1.0	3.6	5.0	2.0	7.3	10.0
carbonate	22.7	40.0	10.0	22.7	40.0	10.0
silicon	5.0	51.0	10.0	5.0	51.0	10.0
iron	0.5	2.7	5.0	1.0	5.3	10.0
minor element solution	-	-	1.0	-	-	1.0

Table 7

Rodhe's No. VIII (R-VIII) Culture Solution*

Element	mg of element/l	Compound	mg of compound/l	mg/ml in stock solution	ml of stock solution/l of medium
calcium	14.7	$Ca(NO_3)_2 \cdot 4H_2O$	60.0	10.0	6.0
magnesium	1.0	$MgSO_4$	5.0	1.0	5.0
sodium	7.5	$Na_2SiO_3 \cdot 9H_2O$	20.0	1.0	20.0
potassium	2.2	K_2HPO_4	5.0	1.0	5.0
sulphur	0.84	$MgSO_4$	5.0		
nitrogen	10.2	$Ca(NO_3)_2 \cdot 4H_2O$	60.0		
phosphorus	0.89	K_2HPO_4	5.0		
iron	0.18	ferric citrate citric acid	1.0 1.0	0.1 0.1	10.0
manganese	0.01	$MnSO_4 \cdot H_2O$	0.03	0.1	0.3
silicon	4.6	$Na_2SiO_3 \cdot 9H_2O$	20.0		

*Rodhe, Wilhelm (66, p.57)

When preparing the Rodhe VIII medium, the stock solution for the modified G-10 medium was used; 10 cc of this stock delivered 5.0 mg/l of silicon.

Table 8

Summary of the Concentrations of the Elements Used in the Various Media in Parts per Million

Element	C-10	R-VIII	Ger. Mod. of C-10	G-10	G-10 (Modified)	M-0
nitrogen	6.8	10.2	6.8	20.4	20.0	15.0
sulphur	3.25	0.84	3.3	1.65	1.0	2.0
phosphorus	1.78	0.89	1.8	0.90	1.0	1.0
potassium	4.47	2.2	4.5	2.25	2.0	2.0
magnesium	2.56	1.0	2.5	0.50	0.50	1.0
calcium	9.7	14.7	9.8	0.98	1.0	2.0
carbonate	22.7	-	22.7	22.7	22.7	22.7
silicon	6.98	4.6	5.7	5.7	5.0	5.0
iron	0.63	0.18	0.56	0.56	0.5	1.0
manganese	-	0.01	0.02	0.02	0.02	0.02

after they had reached room temperature and left until they were inoculated.

In making transfers, standard bacteriological techniques were rigidly followed. Approximately one ml of inoculum was placed in each flask. This was done by means of a sterile glass pipette with a small rubber bulb at one end. The inocula were not, however, of consistent amounts due to the filamentous nature of the alga.

E. Cultural Conditions

1. University of Wisconsin

At the University of Wisconsin, the alga was grown in a culture room maintained at a constant temperature of 25° C (77° F) by means of a refrigerator compressor unit. These cultures were grown under constant illumination provided by two cool, white light fluorescent tubes per shelf. The average light intensity at the level of the shelf was approximately 125 foot candles. The flasks were covered with three layers of cheese cloth which provided a more diffuse and even illumination. The cheese cloth reduced the average light intensity to approximately 100 foot candles.

Experimental cultures were carried in 250 ml Erlenmeyer flasks plugged with cotton; each flask contained 100 ml of medium. Each set of conditions within an

experiment was represented by four flasks, three of which were subsequently harvested. In addition, each experiment was accompanied by a series of flasks containing the prescribed G-10 medium which was known as the control series.

Stock cultures were grown in Erlenmeyer flasks and were transferred about once a week to maintain a vigorous source of inoculum. These cultures were grown under the same physical conditions as the experimental cultures, but were maintained in 125 ml Erlenmeyer flasks, each containing 75 ml of the G-10 medium.

2. Oregon State College

Cultures of the alga (1032) were transferred to Corvallis in small, acid-washed, sterile vials with screw caps. These vials were air mailed from Madison, and upon arrival were kept in a cool location of low light intensity until they were transferred to flasks and illuminated.

Professor Ivan Pratt of the Zoology Department kindly provided a culture room. Continuous illumination was maintained by means of two slim-line fluorescent tubes per culture shelf. The average light intensity reaching the shelf level (ca. 100 f.c.) approximated the intensity previously used.

An attempt was made to maintain a constant temperature in the culture room by means of an air-conditioner. Due to mechanical idiosyncrasies encountered, three temperature conditions were involved in the experimental work:

1. Variable temperatures from ca. 10° C (50° F) to ca. 27° C (80° F)
2. A constant temperature maintained at 15-16° C (60° F)
3. A constant temperature maintained at 21° C (70° F)

During the course of this study, every effort was made to maintain a constant temperature, but the difficulties involved were never overcome.

Conditions other than those mentioned were maintained as they previously were at the University of Wisconsin.

F. Harvesting the Alga

Preliminary experiments conducted by Gerloff (30, p.28) and the writer indicated that the alga obtained maximum growth as measured by the dry weight technique in approximately three weeks. After three weeks, there was no detectable decrease in growth for four to five days. As a result, harvests were made as close to the twenty-first day as possible, but never before 21 days had elapsed.

Prior to harvesting the alga, the pH of each flask was recorded. For each condition within an experiment, three of the four flasks (each containing 100 ml of medium) were harvested. Concomitantly, the control series for the experiment was harvested.

Initial difficulties were encountered in obtaining the displacement of the alga from the medium during centrifugation. Dr. R. I. Evans of the University of Wisconsin suggested using a wetting compound dispensed by the Central Scientific Company (No. 73860-B) which had been employed with some success in other experiments. Two drops of this compound added to 100 ml of medium sufficed to displace the alga during centrifugation. It was unnecessary to add the wetting agent during the second centrifugation. This procedure proved to be unsatisfactory as the high degree of cohesion often caused the loss of the alga during decantation. A number of other compounds were tried, and satisfactory floculation was obtained by using aluminum sulphate which had been recommended by Dr. G. C. Gerloff. Seventy-five ppm of $Al_2(SO_4)_3$ were added to each 100 ml aliquot of the medium, and there was no necessity to add an additional increment during the second centrifugation. In all experiments for which data have been reported, this compound was used.

The addition of aluminum sulphate effected a displacement of the pH to approximately 5.5. After the aluminum sulphate had been added, the flasks were allowed to stand for about 15 minutes. The culture in each flask was divided and placed into two plastic centrifuge tubes containing half of the medium and a portion of the alga. The flasks were then scrubbed with a rubber-policeman and rinsed with distilled water into the centrifuge tubes. The tubes were centrifuged for 10 minutes at a speed of 3,200-3,600 r.p.m.; the speed depending upon the number of tubes in the centrifuge. The supernatant was decanted, the alga washed in distilled water, and centrifuged a second time for 10 minutes.

After the second centrifugation, the supernatant was decanted, and the contents of each flask were washed into a glass, tared weighing bottle with distilled water. The centrifuge tubes were scrubbed with a rubber-policeman and the scrapings washed into the weighing bottle. These bottles were then placed in a drying oven. At Wisconsin, the alga was dried at 65° C in a forced-draft oven, and at Oregon State a temperature of 95° C was used.

During the initial stages of this work, the alga was first oven dried, then placed into a dessicator, weighed, replaced in the oven, etc. until the bottles reached a constant weight. The results of this procedure indicated

that 18-20 hours was sufficient time to evaporate the water, and the constant weight method was discarded; the weights were recorded after the bottles had cooled in the dessicator.

The results for each series within an experiment are recorded here as an average of the contents of the three flasks harvested, and expressed as a percentage of the control series.

EXPERIMENTAL RESULTS

A. Preliminary Investigations of Cultural Conditions

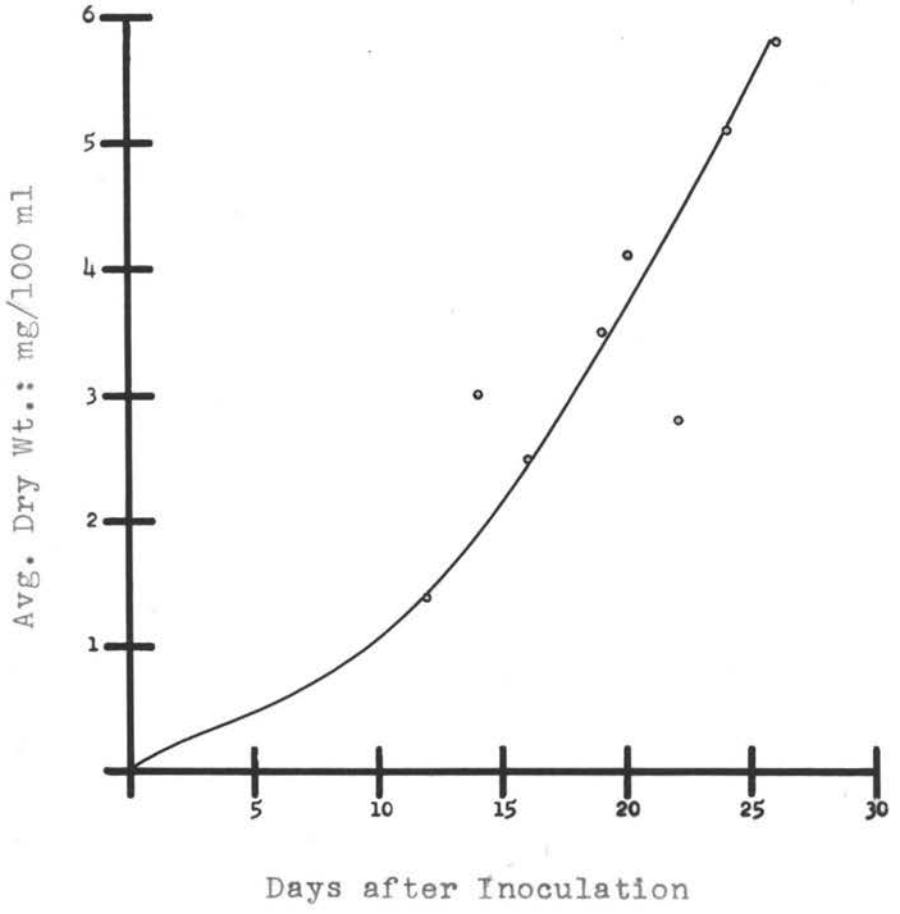
Before the nutrient requirements of the alga could be ascertained, it was necessary to establish the length of time necessary for maximum growth to occur under cultural conditions. To determine the pattern of growth, a large number of flasks were inoculated with strain 1032 and harvested periodically. The first harvest was made 12 days after inoculation, and the last harvest 26 days after inoculation.

These results (Figure 1) indicated poor growth and probably to some extent inconsistent growth. Several repeated attempts were made, all of which resulted in poor and erratic growth. In these experiments, the alga often became chlorotic and coagulated.

The effects of aeration were investigated in attempting to stimulate the growth of 1032. The organism was inoculated into large culture tubes through which compressed air was bubbled. These tubes contained 150 ml of the G-10 medium to which two ml of inoculum had been added. These tubes were placed in a vertical position and illuminated from the side by means of a portable fluorescent light bank. These cultures were

Figure 1

Initial Growth of 1032 at 25° C



eventually discarded due to obvious poor growth and the chlorotic appearance of the alga.

To further ascertain the effects of aeration, a series of flasks containing G-10 medium were inoculated with 1032 and placed on a shaker. Illumination was provided by light from fluorescent tubes reaching the flasks from above with an intensity comparable to that used in other experiments. The shaker was operated continuously and was constantly illuminated. After about two and one-half weeks, there being an almost complete lack of growth, these flasks were discarded.

In all of the preceding experiments, the flasks were covered with three layers of cheese cloth. The light intensity at these flasks was approximately 100 foot candles. A series of flasks containing the G-10 medium were inoculated with 1032 to evaluate growth as it was affected by variation in intensity of light. In this experiment, five series were maintained. One series of flasks was exposed to direct illumination; the remaining four series were covered with from one to four layers of cheese cloth. Those flasks which were left uncovered and those covered with one and two layers of cheese cloth became chlorotic during the course of growth; the greatest degree of chlorosis was experienced by the naked flasks, and chlorosis decreased with each additional layer of

cheese cloth. Those flasks which were covered with three and four layers of cheese cloth maintained a healthy green color during the course of the experiment. Empirical observation indicated that the best growth occurred in those flasks which were covered with three layers of cheese cloth and illuminated at a light intensity of about 100 foot candles. Consequently, in all the following experiments, three layers of cheese cloth were placed over the flasks.

In attempting to increase the growth of 1032, the G-10 medium was enriched by tripling the nitrogen concentration (to 61.2 ppm) and increasing the phosphorus concentration twofold (1.8 ppm). These flasks were carried in conjunction with a series of control flasks. Although growth was slight in both series, the increased concentration of nitrogen and phosphorus apparently introduced an inhibition as indicated by the following results:

	Avg. dry wt. mg/100 cc
G-10	7.1
3X N; 2X P	2.8

Throughout a period of four months, the alga (both strains) had been continuously subcultured about once every week. The inoculum was always taken from those

flasks which displayed the best growth. After four months, growth of strain 1032 had improved considerably, and another growth experiment was initiated using the G-10 medium. Strains 1032 and 1062 were carried concomitantly and harvested periodically during the course of 29 days. The results (Figure 2) revealed that the rate of growth of strain 1032 approached that obtained by Gerloff using Anacystis cyanea (31, pp.26-32) under similar cultural conditions. Conversely, the growth of 1062 was very poor in relation to the growth of strain 1032. These results again showed that at least three weeks are necessary for good growth to occur. Inasmuch as the growth of strain 1062 had always falled considerably below the growth of strain 1032, the former was then discarded as possible experimental material.

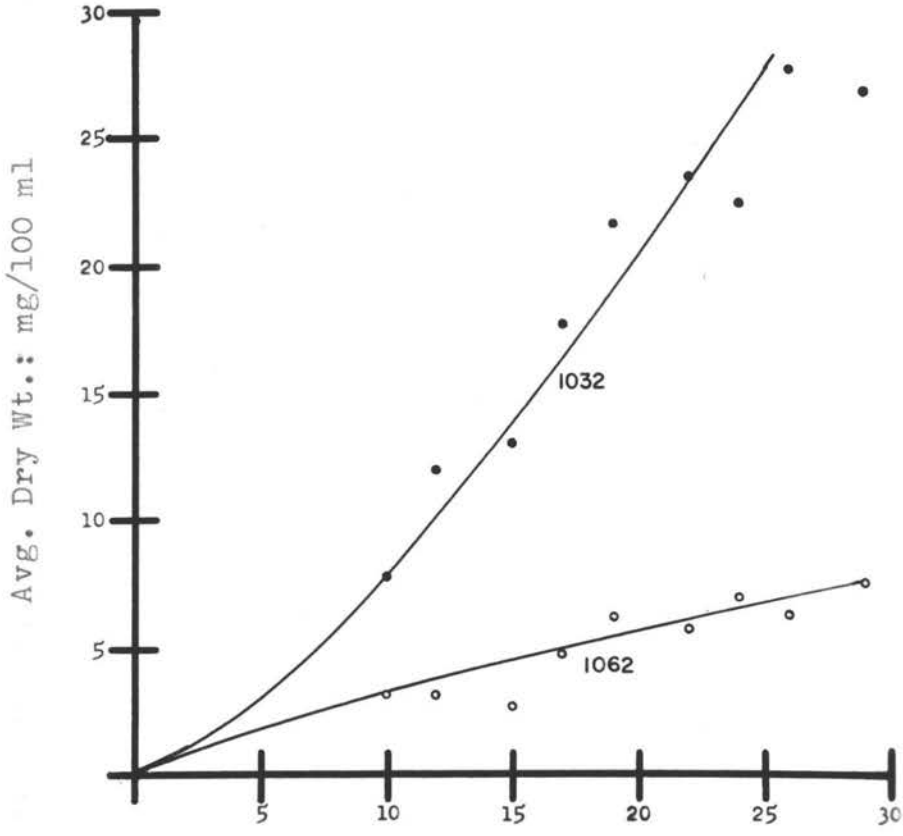
B. Growth of Aphanizomenon as Related to Major Nutrients

1. Nitrogen

Two experiments were designed to determine the relationship of nitrogen to the growth of strain 1032. The first was to determine the effect of various concentrations of nitrate nitrogen, and the second to determine the effects of nitrogen from various sources. In the first experiment, a series of six different concentrations of nitrogen ranging from 0.0 to 25.0 ppm were supplied as

Figure 2

Comparison of the Growth of 1032 and 1062 at 25° C



sodium nitrate in increments of 5.0 ppm. The alga was grown at 25° C, and harvested after 23 days. Maximum growth was obtained at a nitrogen concentration of 15.0 ppm, with apparent retardation of growth at higher concentrations (Table 9). Although the 10.0 and 15.0 ppm series resulted in a higher dry weight content than the 20.0 and 25.0 ppm series, the former were chlorotic at the time of harvest. Consequently, 20.0 ppm of nitrogen were retained in the basic medium. If growth were to increase as a result of stimulation induced by other changes in the medium, concentrations of nitrogen below 20.0 ppm could possibly limit this growth. Nitrogen at 25.0 ppm resulted in neither an increase nor decrease in growth, nor did these cultures display a more healthy appearance.

Table 9

The Effect of the Concentration of Nitrate Nitrogen Upon the Alga Grown at 25° C, and Harvested After 23 Days.

ppm of nitrogen	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.5	no growth	0.0
5.0	8.4	16.0	65.0
10.0	9.1	26.2	106.0
15.0	9.7	29.6	120.0
20.0 (C)	9.7	24.6	100.0
25.0	9.6	24.1	98.0

(C) = Control

The second experiment was concerned with the effects of nitrogen from various sources as related to the growth of the alga. In all of the series within this experiment, nitrogen was maintained at a concentration of 20.0 ppm. In this experiment, four sources of nitrogen were supplied in the following manner:

Sodium nitrate (Control)	$NaNO_3$
Sodium nitrite	$NaNO_2$
Ammonium chloride	NH_4Cl
Ammonium chloride; added separately after autoclaving	
Ammonium nitrate; added separately after autoclaving	NH_4NO_3

The ammonium compounds that were autoclaved separately were added under aseptic conditions. This procedure was used to insure against a loss of ammonia from the alkaline medium. The alga was grown at a constant temperature of 21° C, and harvested after 22 days of growth.

Maximum growth occurred when the source of nitrogen was a nitrate (Table 10). The presence of nitrite in the medium was not detrimental to the growth of the alga. Final conclusions regarding the physiological effects of this ion, however, must be postponed, as it is remotely possible that the nitrite may have been converted to nitrate by bacterial action. Poor growth obtained in those series in which nitrogen had been supplied as NH_4Cl

was probably due, at least in part, to the pH of the medium. At the time of the harvest, the pH averaged 6.6 (NH_4Cl) and 6.2 (NH_4Cl added after autoclaving). The reduced growth obtained in the series containing NH_4NO_3 may likewise have been due to an unfavorable pH level of the medium. This may have occurred during part of the growth period if the alga absorbed ammonium ions in preference to nitrate ions. If this series had been allowed to grow for a longer period of time, the dry weight might have approximated that obtained in the control series. The pH of the medium in both the $NaNO_3$ and NH_4NO_3 series was 9.2 at the time they were harvested. There is also a possibility that the ammonium ion per se exerted a toxic effect. If the alga absorbed this ion more rapidly than it was utilized, toxic effects could have become evident.

Table 10

The Effect of Various Sources of Nitrogen Upon the Alga Grown at 21° C, and Harvested After 22 Days.

Nitrogen source	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
$NaNO_3$ (C)	9.2	32.5	100.0
$NaNO_2$	9.1	29.7	91.5
NH_4Cl	6.6	7.9	24.3
NH_4Cl added separately	6.2	8.1	24.7
NH_4NO_3 added separately	9.2	24.6	75.6

At the beginning of this experiment, flasks were prepared for possible initial pH adjustments. The initial pH's within these series did not seem to warrant such an adjustment, being about 8.6 in all cases.

2. Phosphorus

Two experiments were conducted to determine the effect of the concentration of phosphorus upon the growth of the alga. In both of these experiments, phosphorus was supplied as sodium monophosphate (Na_2HPO_4).

Table 11

The Effect of the Concentration of Phosphorus Upon the Alga Grown at 25° C, and Harvested After 23 Days.

ppm of phosphorus	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.1	7.9	29.6
0.5	9.4	25.2	93.4
1.0 (C)	9.2	27.0	100.0
1.5	9.3	27.7	102.5
2.0	9.2	28.9	110.5
2.5	9.3	28.2	108.2

In the first experiment six series of phosphorus concentrations ranging from 0.0 to 2.5 ppm in increments of 0.5 ppm were established. The alga was grown at a constant

temperature of 25° C, and harvested after 23 days of growth. Little difference in growth among the series containing at least 0.5 ppm of phosphorus was evident (Table 11). In addition, the series to which no phosphorus had been added displayed an appreciable amount of growth. This might be explained on the basis of accumulated phosphorus in the cells of the inoculum; the amount sufficing for a small increment of growth. Another possible explanation resides in the presence of a small amount of phosphorus carried over in the medium with the inoculum. It may be that phosphorus from both these sources manifested its effect. These results did not, however, indicate a level at which phosphorus might exert a limiting effect upon the growth of the alga.

To ascertain the minimum level of phosphorus necessary to obtain optimal growth, seven concentrations of phosphorus ranging from 0.0 ppm to 0.5 ppm in increments of 0.1 ppm were established. In addition, a control series with a phosphorus concentration of 1.0 ppm was maintained. The alga was grown at 25° C, and harvested after 21 days. The results indicated that at least 0.2 ppm of phosphorus ought to be maintained in the medium for good growth (Table 12). However, initial chlorosis was evident in all of the series with the exception of the control. This would suggest that a concentration of phosphorus of at least 0.5 ppm in the

medium would be necessary to insure against a limiting effect being exerted by this nutrient.

Table 12

The Effect of the Concentration of Phosphorus Upon the Alga Grown at 25° C, and Harvested After 21 Days.

ppm of phosphorus	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.1	4.3	14.9
0.1	8.6	21.2	78.5
0.2	9.0	24.3	89.8
0.3	9.0	24.4	90.5
0.4	9.1	26.3	97.4
0.5	9.1	26.8	99.3
1.0 (C)	9.4	27.0	100.0

3. Potassium

To determine the effect of the concentration of potassium upon the growth of the alga, six series were established at the following concentrations in ppm of potassium: 0.0, 0.4, 0.8, 1.6, 2.25 (control) and 4.8. The alga was grown at 25° C, and harvested after 24 days. Maximum growth of the alga was achieved when the concentration of potassium was 1.6 ppm (Table 13). An increase in concentration above 1.6 ppm did not result in an increase in growth, whereas a reduction below this concentration

resulted in a marked decrease in the growth of the alga. There were no indications of a toxic effect at the higher concentrations of potassium. As in the case of phosphorus, the appreciable amount of growth in the series to which no potassium had been added may possibly be explained on the basis of accumulated potassium in the algal cells and/or potassium carried over in the inoculum.

Table 13

The Effect of the Concentration of Potassium Upon the Alga Grown at 25° C, and Harvested After 24 Days.

ppm of potassium	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.3	11.0	35.2
0.4	8.5	17.8	55.4
0.8	8.7	23.6	75.7
1.6	9.1	32.3	103.5
2.25 (C)	9.2	31.2	100.0
4.8	9.2	30.9	99.0

4. Sulphur

To determine the effect of the concentration of sulphur upon the growth of the alga, six sulphur concentrations were established. These concentrations varied from 0.0 ppm to 3.0 ppm in increments of 0.5 ppm, with the series at 2.5 ppm being omitted. Sulphur was supplied as

sodium sulphate. The alga was grown at 25° C, and was harvested after 23 days of growth.

Maximum growth occurred when the sulphur concentration exceeded 1.0 ppm (Table 14). At concentrations greater than 1.5 ppm, no appreciable increment of growth was evident, nor was an inhibition noted at these higher concentrations.

Table 14

The Effect of the Concentration of Sulphur Upon the Alga Grown at 25° C, and Harvested After 23 Days.

ppm of sulphur	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.1	5.1	18.1
0.5	8.0	18.2	65.8
1.0	8.3	24.5	88.5
1.5 (C)	8.4	27.7	100.0
2.0	8.5	29.6	106.9
3.0	8.4	27.7	100.0

5. Magnesium

To ascertain the effect upon growth of the alga by variations in the concentration of magnesium supplied as magnesium chloride, the following concentrations in ppm of magnesium were established: 0.0, 0.05, 0.1, 0.2, 0.4, and 1.0. The alga was grown at 25° C, and harvested after 22 days of growth.

Maximum growth occurred at 1.0 ppm (Table 15). However, the increase in growth which occurred at concentrations greater than 0.2 ppm was rather slight in relation to the relatively large increments of magnesium necessary to incur these results. These data indicate that magnesium is apt to exert a limiting effect when the concentration in the medium falls below 0.2 ppm.

Table 15

The Effect of the Concentration of Magnesium Upon the Alga Grown at 25° C, and Harvested After 22 Days.

ppm of magnesium	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.1	5.1	18.4
0.05	8.4	8.1	29.5
0.1	8.3	12.0	44.2
0.2	9.2	25.8	93.9
0.4 (C)	9.4	27.2	100.0
1.0	9.3	30.8	113.1

6. Iron

The effects of iron concentration and source upon the growth of the alga were studied in three experiments. To determine the effect of various concentrations of iron upon the growth of the alga, iron was supplied in the medium as ferric citrate with citric acid as a stabilizing agent;

six concentrations of iron were supplied in the following series: 0.0, 0.11, 0.28, 0.56, 0.84, and 1.12 ppm. A freshly prepared iron solution was used for this experiment. The alga was grown at 25° C, and harvested after 22 days of growth.

Table 16

The Effect of the Concentration of Iron, Supplied as Ferric Citrate, Upon the Alga Grown at 25° C, and Harvested After 22 Days.

ppm of iron	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.00	8.1	no growth	0.0
0.11	8.7	15.8	58.5
0.28	9.3	26.6	98.6
0.56 (C)	9.4	27.0	100.0
0.84	9.4	30.0	111.0
1.12	9.4	30.5	113.0

Maximum growth of the alga was achieved when iron was supplied at a concentration of 1.12 ppm (Table 16). There was, however, very little, if any, difference between the 0.84 and 1.12 ppm series. In addition, the increase in growth that was obtained by increasing the concentration above 0.28 ppm was slight in relation to the large increase of iron. Iron in high concentrations may exert a toxic effect upon the growth of the alga, but these levels were not reached in this experiment.

In some of the more recent cultural works (42, pp.152-170), iron has been supplied to the medium in a chelated form using ethylenediaminetetraacetic acid (hereafter EDTA). Those results indicated that this compound was nontoxic to the growth of their experimental organism. To compare the effect of iron supplied as ferric citrate and as sodium ferric ethylenediaminetetra-acetate (hereafter NaFe-EDTA), five series were erected under the following conditions:

a. One series containing no iron
b. Two series containing iron supplied as ferric citrate; one with 0.5 ppm of iron, and one with 1.0 ppm of iron
c. Two series containing iron supplied as NaFe-EDTA; one with 0.5 ppm of iron and one with 1.0 ppm of iron

The alga was grown at 16° C, and harvested after 22 days of growth.

Very little difference among the series containing iron supplied as ferric citrate and that containing iron supplied as NaFe-EDTA at a concentration of 0.5 ppm was observed (Table 17). However, in the series containing iron supplied as NaFe-EDTA at a concentration of 1.0 ppm, growth was depressed approximately 50 percent. The alga in this series, too, was chlorotic at the time of harvest.

Table 17

The Effect of the Concentration of Iron Supplied as Ferric Citrate and NaFe-EDTA Upon the Alga Grown at 16° C, and Harvested After 22 Days.

ppm of iron	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.1	7.0	24.2
0.5 Fe-Cit (C)	9.5	28.9	100.0
1.0 Fe-Cit	9.5	31.7	109.6
0.5 NaFe-EDTA	9.5	29.8	103.0
1.0 NaFe-EDTA	8.7	15.3	53.0

To determine the minimum requirements of iron supplied as NaFe-EDTA and to find a possible explanation of the inhibition of growth noted in the previous experiment, the following series were established:

a. One series containing no iron

b. Five series containing iron supplied as NaFe-EDTA at concentrations of 0.01, 0.10, 0.25, 0.50, and 0.75 ppm

c. One series containing 0.5 ppm of iron supplied as ferric citrate

d. One series containing 0.5 ppm of iron as ferric citrate plus 6.0 ppm of EDTA; this is the concentration of EDTA delivered when 1.0 ppm of iron is supplied as NaFe-EDTA

The initial pH of all series was approximately the same; consequently, no pH adjustments were necessary. The alga was grown under fluctuating temperature conditions; the variations were, however, not great, and all cultures experienced the same variations. The cultures from this experiment were harvested after 23 days of growth.

Table 18

The Effect of the Concentration of Iron Supplied as NaFe-EDTA Upon the Alga Grown at 21° C, and Harvested After 23 Days.

ppm of iron	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.00	8.0	no growth	0.0
0.01	8.6	14.9	46.6
0.10	9.3	31.3	97.8
0.25	9.2	29.8	93.2
0.50	8.7	19.2	60.1
0.75	8.7	17.7	55.3
0.50 Fe-Cit (C)	9.5	32.0	100.0
0.50 Fe-Cit + EDTA	8.2	9.2	28.2

Good growth was produced when 0.1 ppm of iron was supplied as NaFe-EDTA and was comparable to that obtained when an iron concentration of 0.5 ppm supplied as ferric citrate was used (Table 18). Also, at this concentration of iron supplied in a chelated form, no chlorosis was

evident. At increasing concentrations greater than 0.1 ppm there was a progressive decrease in the growth of the alga. Growth was also considerably reduced in the series to which 6.0 ppm of EDTA was added.

No explanation has been found for the discrepancy between the results obtained in this experiment with iron supplied at a concentration of 0.5 ppm and the results which were obtained in the immediately preceding experiment. In the second experiment, growth was 60 percent of the control, and in the first experiment 103 percent of the control. Two additional experiments were performed using 0.5 ppm of iron supplied as NaFe-EDTA; these results closely approximating those reported in the present experiment. The cause of this discrepancy evidently resides within an experimental error.

The results of this experiment indicated that with concentrations greater than 0.2 ppm of iron incipient iron toxicity was reached. These same results also indicated that the alga absorbed the iron in the complexed form rather than in the ionic form. It seems apparent that a considerable amount of iron must be rendered unavailable for metabolic purposes when supplied in the form of ferric citrate. The reduction in growth experienced by the series supplied with EDTA may possibly be explained on

the basis of a toxic amount of iron supplied to the alga and/or toxicity or unavailability of some of the other essential nutrients.

It would seem advisable to supply iron in the form of NaFe-EDTA at dilute concentrations in the basic medium used for the growth of the alga. This would eliminate the necessity of frequently preparing fresh iron solutions, and a more constant concentration of iron could be maintained in the medium.

7. Calcium

As was previously mentioned, calcium has been regarded as unessential in the nutrition of some chlorophycean organisms as well as one blue-green planktont. To ascertain the effect of the concentration of calcium upon the growth of the alga, six series of calcium concentrations were established ranging from 0.0 ppm to 1.6 ppm in increments of 0.4 ppm with an additional series at 2.4 ppm. The cultures in this experiment were grown at 25° C, and harvested after 22 days of growth.

The results indicated that in the nutrition of this alga calcium is an essential element. Maximum growth was obtained in the series containing 0.8 ppm of calcium, and additional increments were not paralleled by an increase in growth (Table 19). No inhibition of growth was

observed at concentrations greater than that necessary for the maximum expression.

Table 19

The Effect of the Concentration of Calcium Upon the Alga Grown at 25° C, and Harvested After 22 Days.

ppm of calcium	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	8.3	6.6	25.8
0.4	8.6	19.6	76.6
0.8 (C)	9.0	25.6	100.0
1.2	9.0	23.5	91.9
1.6	9.1	25.5	99.8
2.4	9.0	26.8	104.6

8. Strontium

Chemically, strontium reacts very similarly to calcium. To determine the replaceability of calcium by strontium as an essential element in the nutrition of Aphanizomenon flos-aquae, two media were used; the G-10 medium and the R-VIII medium. In the G-10 medium, calcium chloride was replaced by strontium chloride ($SrCl_2$), and in the R-VIII medium, strontium nitrate ($Sr(NO_3)_2$) replaced calcium nitrate. Within this experiment five series were established:

a. G-10 medium (Control); 1.0 ppm of Ca as $CaCl_2$

b. G-10 medium; neither Ca nor Sr

c. G-10 medium; 1.0 ppm of Sr as $SrCl_2$

d. R-VIII; 14.7 ppm of Ca as $Ca(NO_3)_2$

e. R-VIII; 14.5 ppm of Sr as $Sr(NO_3)_2$

The alga in this experiment was grown at 16° C, and harvested after 24 days of growth.

Table 20

The Effect of Calcium and Strontium Upon the Alga Grown at 16° C, and Harvested After 24 Days.

Series	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
G-10; 1.0 ppm Ca (C)	9.4	28.8	100.0
G-10; no Ca or Sr	8.5	13.8	48.8
G-10; 1.0 ppm Sr	8.5	17.6	61.2
R-VIII; 14.7 ppm Ca	7.7	13.5	47.9
R-VIII; 14.5 ppm Sr	7.5	no growth	0.0

The best growth of the alga occurred in those media to which calcium had been added (Table 20). There was very little difference in growth obtained in the G-10 medium containing neither calcium nor strontium and in this same medium to which 1.0 ppm of strontium had been added. However, these data did suggest that calcium was replaceable by strontium to a limited extent. The R-VIII medium had previously not proved satisfactory for measuring the growth of the alga, although the organism was able to subsist in this medium. The complete suppression of growth

in the R-VIII medium to which strontium had been added may have been due to one or both of the following factors: (1) a toxic concentration of strontium had been established or (2) an inhibition resulting from the 24.1 ppm of nitrogen necessary to establish the desired concentration of strontium. The former explanation seems to be supported by the fact that the G-10 medium containing 25.0 ppm of nitrogen supported a reduced growth, but growth was not completely suppressed at this concentration.

A series of cultures were established in an attempt to select a strain of the alga able to grow well under conditions in which the calcium was replaced by strontium. Approximately a dozen flasks were prepared using the G-10 medium to which 1.0 ppm of strontium was added as strontium chloride. Under these conditions the alga was carried through four transfers. Each time the flask resulting in the best growth (empirical observation) was used as a source of inoculum. Growth progressively decreased in each succeeding transfer, and after the fourth transfer, the cultures were discarded as a result of extremely poor growth.

9. Silicon

Gerloff (29, pp.835-840; 31, pp.26-32) reported that no evidence of added nutritive value was obtained by the

inclusion of silicon in his medium. Sodium silicate was added to effect an alkaline condition in the medium. To ascertain the applicability of Gerloff's conclusions with relation to A. flos-aquae, six series of various silicon concentrations were prepared between 0.0 ppm and 10.0 ppm of silicon in increments of 2.0 ppm. The cultures were grown at 25° C, and harvested after 22 days of growth.

Table 21

The Effect of the Concentration of Silicon Upon the Alga Grown at 25° C, and Harvested After 22 Days.

ppm of silicon	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
0.0	9.4	28.5	105.4
2.0	9.3	28.3	104.8
4.0	9.3	22.8	84.5
6.0 (C)	9.3	27.0	100.0
8.0	9.3	27.2	100.6
10.0	9.0	23.8	88.2

In order to obtain maximum growth, silicon need not be added to the medium (Table 21). Silicon, at least above the level existing as a contaminant, was neither necessary in the nutrition of this alga nor did its absence effect a deleterious pH level in the medium. The results obtained in the 4.0 ppm series are difficult to explain, but on the basis of the growth obtained at higher and lower

concentrations, it seemed quite reasonable that it arose from some experimental error. It may, too, have been due to an idiosyncrasy of growth of the organism. These data also suggested that silicon may induce an inhibition or become toxic to the growth of the alga at higher concentrations. This observation seemed justifiable on the basis of the chlorotic appearance of the alga observed in those series containing 8.0 and 10.0 ppm of silicon; the chlorosis was seemingly more pronounced in the 10.0 ppm series. Another possibility may have been that a silicate-phosphate antagonism was established.

10. Silicate and Carbonate

To determine the effect of silicate and carbonate as related to the growth of the alga, eight series using sundry combinations of silicate and carbonate were established:

pH of medium at inoculation	Conc. of carbonate	Conc. of silicate
6.1	none	none
7.8	X	none
7.4	none	X
8.3	2X	X
8.2	X	2X
8.0	X	X
7.8	1/2X	X
8.0	X	1/2X

In the above chart, X equals the concentration found in the G-10 medium. The alga was grown at 16° C, and harvested after 22 days of growth.

Table 22

The Effect of Silicate and Carbonate* Upon the Alga Grown at 16° C, and Harvested After 22 Days.

CO_3 conc.	SiO_3 conc.	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
none	none	6.8	no growth	0.0
X	none	9.3	18.5	61.7
none	X	9.2	29.0	96.7
2X	X	9.3	27.8	92.8
X	2X	9.2	34.7	115.8
X	X	9.3	30.0	100.0
1/2X	X	9.3	31.5	105.0
X	1/2X	9.3	29.6	98.6

In the absence of both silicate and carbonate, growth of the alga was completely suppressed (Table 22). The omission of carbonate from the medium did not seem to reduce growth. The data obtained in this experiment tended to indicate that an omission of silicon from the medium would seriously reduce the growth of the alga. This is contrary to those data presented in the preceding

*X concentration gives 13.6 ppm of silicate and 22.7 ppm of carbonate.

experiment. Another point contrary to the results obtained in the preceding experiment is the excellent growth obtained with a twofold increase in the silicon concentration (10.0 ppm). The preceding experiment tended to indicate an inhibition of growth at this concentration. These conflicting data were probably due to an idiosyncrasy in the growth of the organism. These data did indicate, however, that either silicate or carbonate must be present in the medium to effect a favorable alkaline environment. Apparently no direct nutritive value was obtained from either of these ions.

C. Effect of Various Media

1. The G-10 Medium

Following the completion of the experiments relating to the effects of concentration of the major nutrient minerals, a modification of the G-10 medium was devised which is hereafter referred to as the M-0 medium (Table 6) in which the composition was based on the optimum concentration for each nutrient as determined by the previous experiments. A comparative growth study was made to compare the efficacy of these two media. The alga was grown at 16° C for a total of 30 days with periodic harvests from the two media.

Maximum growth was obtained more quickly in the M-O medium than in the G-10 medium (Figure 3). At the end of 30 days, however, both of these media seemed to be equally efficient in supporting growth. At the termination of this period, the alga in both media had become chlorotic. Chlorosis was evident in the M-O medium before the alga in the G-10 medium had become noticeably chlorotic. Apparently the more rapid the growth, the more quickly the alga depleted the medium and became chlorotic. This probably indicated that some or all of the nutrients became limiting in the M-O medium before these nutrients exerted a limiting effect in the G-10 medium. The rapidity of the initial growth in the M-O medium may have been due to some of the nutrients being supplied at a concentration below the inhibitory level (e.g. nitrogen).

To determine the effects of the basic complement of nutrients, seven series were established. These concentrations ranged from 4X to 1/8X; each series containing one-half the concentration of the preceding series. In addition, one series was completely lacking in nutrients. After autoclaving, a considerable amount of precipitate was noted in the 4X series, while the remainder were without an apparent precipitate. The alga was grown at 21° C, and harvested after 24 days of growth.

Figure 3

Comparative Growth Rate of 1032 in the G-10 Medium and in the M-O Medium at 16° C

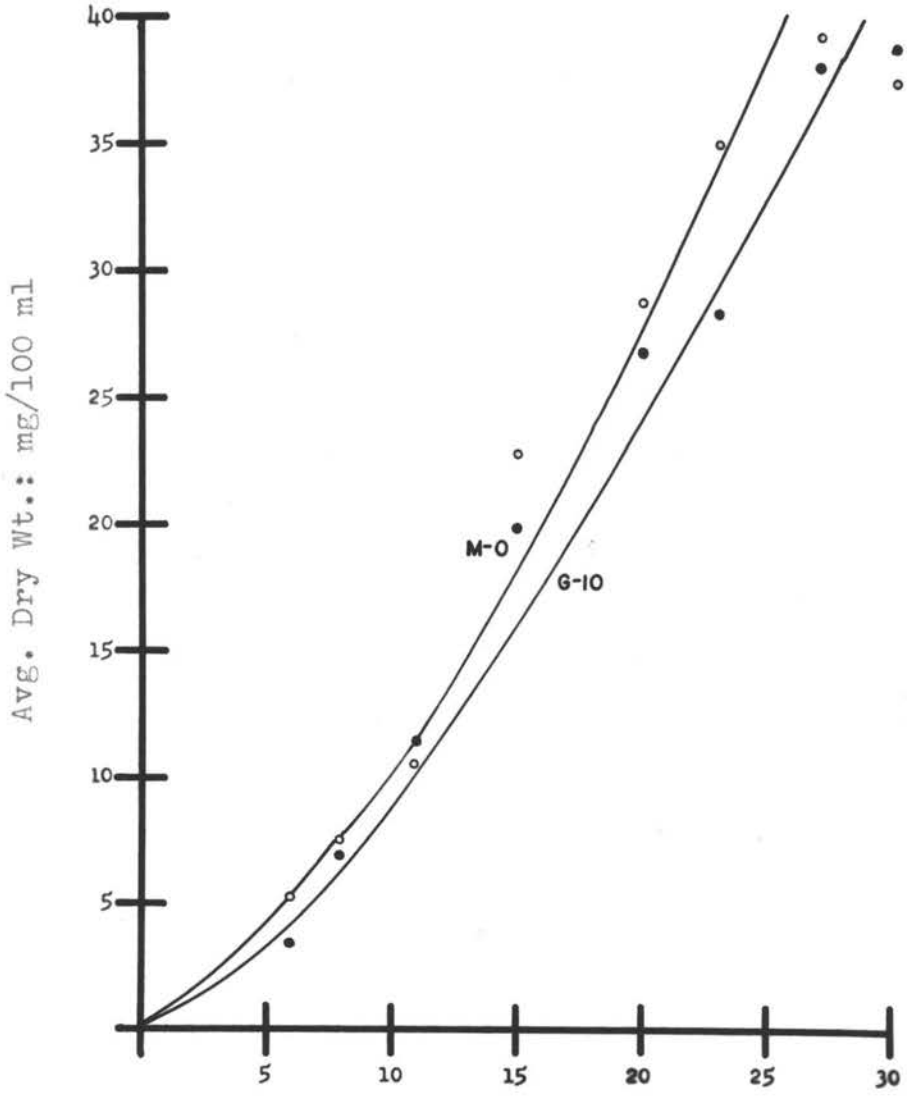


Table 23

The Effect of Varying the Basic Medium Upon the Alga Grown at 21° C, and Harvested After 24 Days.

Conc. of the basic medium	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
4X	9.5	30.6	107.4
2X	9.7	38.7	135.8
X (C)	9.1	28.5	100.0
1/2X	8.4	16.6	57.9
1/4X	7.6	8.0	28.1
1/8X	7.3	6.4	21.2
0X	6.7	no growth	0.0

Approximately comparable growth was obtained in the 4X series as was obtained in the control (Table 23). On the other hand, the 2X series showed a considerable increase in growth over the 4X and X series. Those series receiving concentrations less than the control exhibited a sharp reduction in growth. At the time these series were harvested, the 4X and 2X series revealed no indication of chlorosis. Incipient chlorosis was noted in the X series, and in the remaining series the chlorotic appearance of the alga was pronounced. In addition, the 4X series seemed to be in a state of vigorous growth. This was evidenced by a large accumulation of gas bubbles at the surface of the medium. Empirically, the 4X and 2X series

seemed to exhibit a slower initial growth than the rest of these series. Retardation of growth in these series may have been due to inhibitory concentration of some of the nutrients. At the time of harvest, the inhibitory level of these nutrients had presumably been eliminated, and an additional increment of growth might have been obtained if these cultures had been allowed to continue growth. Inhibitory levels in the 2X series had been eliminated before the time of harvest, resulting in more growth than was noted in the 4X series. The initial pH of the medium in the 1/4X and 1/8X series fell considerably below that of those series having a higher concentration of nutrients. This may account for at least part of the reduced growth in these series.

2. R-VIII Medium

The R-VIII medium is a physiologically balanced medium. This medium (Table 7) differs from the G-10 medium in regard to the concentration of the following major nutrients: (1) a lower nitrogen and iron content (10.2 ppm and 0.18 ppm respectively) and (2) a higher calcium concentration (14.7 ppm).

The first experiment in the study of this medium involved the establishment of the following seven series of variations:

a. G-10 medium; no variation

b. G-10 medium; calcium content increased to 15.0 ppm

c. R-VIII medium; no variation

d. R-VIII medium; no variation, but phosphorus added separately to the medium after autoclaving

e. R-VIII medium; iron increased to 0.5 ppm, and phosphorus added separately

f. R-VIII medium; nitrogen increased to 20.2 ppm (10.2 ppm as $Ca(NO_3)_2$ and 10.0 ppm as $NaNO_3$), and phosphorus added separately

g. R-VIII medium; iron increased to 0.5 ppm, nitrogen increased to 20.2 ppm (as in series F), and phosphorus added separately

Rodhe recommended autoclaving the phosphorus separately since calcium and phosphorus together in the medium may be precipitated as calcium phosphate. A white precipitate was noted in the C series after autoclaving, which was presumably calcium phosphate. In this experiment the alga was grown at 16° C, and harvested after 24 days of growth.

A calcium concentration in the G-10 medium comparable to that found in the R-VIII medium resulted in approximately comparable growth in these two media; growth being depressed to about 60 percent of the control (Table 24). This may have been a direct inhibition of growth by the calcium, or

it may have been the result of secondary effects of the presence of a high calcium concentration in the medium. There also seems to have been very little benefit, if any, derived from adding phosphorus separately to the medium. The increase in nitrogen and iron in the R-VIII medium did not seemingly stimulate growth to any extent. These results, however, are not conclusive. If an increase in the concentration of these nutrients had initiated a favorable action upon the growth of the alga, these results were probably suppressed by the inhibitory calcium concentration.

Table 24

The Effect of Varying the R-VIII Medium Upon the Alga Grown at 16° C, and Harvested After 24 Days.

Series	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
G-10 (C)	9.2	32.5	100.0
G-10; 15 ppm Ca	9.0	19.0	58.4
R-VIII	8.1	14.8	45.6
R-VIII; P separate	8.0	17.0	52.3
R-VIII; 0.5 ppm Fe P separate	8.1	19.4	59.7
R-VIII; 20.0 ppm N P separate	8.6	15.8	48.6
R-VIII; 0.5 ppm Fe 20.0 ppm N P separate	9.0	20.8	64.0

In order to ascertain the limitations supposedly imposed upon the growth of the alga by a relatively high calcium concentration, five series were erected in the following manner:

a. R-VIII; no variation

b. R-VIII; 20.2 ppm of nitrogen (10.2 ppm as $Ca(NO_3)_2$ and 10.0 ppm as $NaNO_3$ with the addition of 10 cc of Na_2CO_3 (G-10 concentration)

c. R-VIII; 20.0 ppm of nitrogen as $NaNO_3$ and 1.0 ppm of Ca as $CaCl_2$

d. R-VIII; 20.0 ppm of nitrogen as $NaNO_3$, 1.0 ppm of calcium as $CaCl_2$, and 0.5 ppm of iron

e. G-10; no variation

The alga was grown at a variable temperature range but with no drastic fluctuations, the average temperature being about 21° C. These cultures were harvested after 22 days of growth.

Inhibition of growth which was apparently imposed by a high calcium concentration can be overcome in the R-VIII medium by a reduction of calcium to the concentration found in the G-10 medium and/or an addition of sodium carbonate to the medium (Table 25). The buffering action of the carbonate may have been responsible for mitigating the deleterious effect of a high calcium content. In addition, with a reduction in the calcium

concentration and with an increase in the concentration of nitrogen and iron, growth in the R-VIII medium was increased to a point comparable to that obtained in the G-10 medium. These data, therefore, indicated that with some modifications, a physiologically balanced medium is capable of supporting growth equaling that supported by a physiologically unbalanced medium.

Table 25

The Effect of Varying the R-VIII Medium Upon the Alga Grown at 21° C (Average) and Harvested After 22 Days.

Series	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
R-VIII	8.1	14.8	46.8
20.2 ppm N; 10 ml Na_2CO_3	9.4	28.2	89.4
20.0 ppm N; 1.0 ppm Ca	9.4	27.2	86.1
20.0 ppm N; 1.0 ppm Ca; 0.5 ppm Fe	9.8	32.9	104.2
G-10 (C)	9.4	31.6	100.0

3. Klamath Lake Mud and Water

Klamath Lake in southern Oregon supports a heavy growth of Aphanizomenon occidentalis. It was deemed desirable to use the mud and water from this lake as the basis of culture media. The mud and water were collected in January, 1956.

This experiment was divided into the following three parts:

1. Media containing an extract from bottom mud
2. Biphasic media prepared by adding bottom mud to sundry media
3. Media employing filtered lake water

The mud extract used in this experiment was prepared in the following manner:

1. Two hundred grams of oven-dried mud were placed in two liters of boiling, glass-distilled water and allowed to steep for 20 minutes.
2. The supernatent was decanted and filtered through celite filter-aid.
3. Chloroform was added to the filtrate which was stored at 2° C until it was used.

Using this mud extract instead of distilled water, the following series were established and inoculated with strain 1032:

a. Extract; no variation
b. Extract; enriched with R-VIII nutrients
c. Extract; enriched with G-10 nutrients

No growth was obtained in any of these series containing mud extract. This may have been due, at least in part, to an inimical effect of the acidic nature (5.7-6.6) of these media.

The biphasic media were prepared in the following manner:

1. One hundred ml of medium were placed into each flask.
2. Five grams of oven-dried mud were added to each flask.
3. These flasks were then autoclaved and allowed to clear before being inoculated.

The following five series were erected using biphasic media and inoculated with 1032:

a. Mud and R-VIII nutrients
b. Mud and G-10 nutrients
c. Mud and Klamath Lake water
d. Mud and Klamath Lake water enriched with R-VIII nutrients
e. Mud and Klamath Lake water enriched with G-10 nutrients

No growth was obtained in any of these flasks containing biphasic media. Again, this may have been due to the acidic nature (6.4-6.7) of these media.

The third phase of this experiment was erected using surface water from Klamath Lake which had been filtered through celite filter-aid. The following seven series were erected and inoculated with 1032:

a. Lake water; no adjustments

b. Lake water enriched with R-VIII nutrients

c. Lake water enriched with G-10 nutrients

d. Lake water enriched with 0.5 ppm of iron as ferric citrate

e. Lake water enriched with 1.0 ppm of phosphorus as sodium phosphate

f. Lake water enriched with 20.0 ppm of nitrogen as sodium nitrate

g. Lake water enriched with 0.5 ppm of iron, 1.0 ppm of phosphorus, and 20.0 ppm of nitrogen

Unfortunately, no control series was included in this experiment; consequently, the C series was used as a reference point (control). In all three phases within this experiment, the alga was grown at a temperature range between 21° C and 27° C, and harvested after 23 days of growth.

A certain amount of nutritive value was obtained from the unenriched lake water (Table 26). These data also indicated that this particular water was lacking in nutrients other than iron, nitrogen, and phosphorus, as an addition of these three nutrients was not sufficient to increase the growth beyond that obtained with the unenriched lake water. A complete set of nutrients did, however, result in excellent growth. The growth in both

the R-VIII and in the G-10 series exceeded that which had heretofore been obtained in either of the media when used unenriched; an increase which amounted to almost 25 percent. It is not known if this stimulus were due to the presence of inorganic or to organic substances in the lake water.

Table 26

The Effect of Klamath Lake Water Upon the Alga Grown Between 21°-27° C, and Harvested After 23 Days.

Series	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
no nutrients added	8.3	5.1	13.2
R-VIII nutrients	9.1	25.8	67.7
G-10 nutrients (C)	10.2	38.1	100.0
0.5 ppm Fe	8.2	9.1	23.7
1.0 ppm P	8.3	6.8	15.8
20.0 ppm N	8.2	6.4	15.9
0.5 ppm Fe; 1.0 ppm P; 20.0 ppm N	8.2	6.3	15.9

D. pH of the G-10 Medium

The pH of the G-10 medium (including most variations) was usually 8.0 to 8.4. As growth ensued in this medium, the pH increased. The amount of growth which took place was to some extent related to the pH, but no close

correlation was evident. The highest pH measurements made at the time of harvest were around 10.0, but the pH usually lay somewhere between 9.0 and 9.4.

Two experiments were performed to relate growth to the pH of the medium. However, neither of these experiments was particularly successful; the difficulties encountered were probably related to the buffered nature of the medium. In both of these experiments, it was not feasible to maintain aseptic conditions as it was necessary to adjust the pH after the medium had been autoclaved. These adjustments were made with dilute sodium hydroxide and hydrochloric acid (each ca. 0.1 N).

To determine the effect of the initial pH of the medium upon the growth of the alga, eight series were established. The initial pH in these series ranged between 5.0 and 11.0 in increments of one pH unit; one series received no initial adjustment. These adjustments took place 36 hours after the flasks had been autoclaved. The alga was grown at 16° C, and harvested after 23 days of growth.

The best growth was obtained in those series in which the initial pH had been adjusted to 10.0 and 11.0 (Table 27). These series very closely approximated the growth in the control series. Poor growth was obtained in the 9.0 series, and no harvestable amount of alga grew

in the 8.0 series. However, the alga in these series did appear to be in a healthy condition, and if these series had been allowed to grow for a longer period, there may have been considerable growth. No observable growth occurred in any of the series below 8.0

Table 27

The Effect of the Initial pH of the G-10 Medium Upon the Alga Grown at 16° C, and Harvested After 23 Days.

Initial pH	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
5.0	7.4	no growth	0.0
6.0	7.4	no growth	0.0
7.0	7.7	no growth	0.0
8.0	7.7	no growth	0.0
9.0	9.5	16.1	57.9
10.0	9.4	27.7	99.7
11.0	9.4	25.8	92.8
no adjustment (C)	9.4	27.8	100.0

To determine the effect upon the growth of the alga of a daily adjustment of the pH, five series were established. These daily adjustments ranged from 9.0 to 12.0 in increments of one pH unit. The first adjustment was made six days after these flasks had been inoculated, and henceforth adjustments were made every 24 hours until

the eighteenth day after inoculation (Table 28). In making the adjustment, the contents of one flask were placed into a beaker, the pH adjusted, and the medium and alga replaced in the flask. In this experiment the alga was grown at a variable temperature range but with no extreme fluctuations. The harvest took place 22 days after inoculation.

Table 28

The Effect of a Daily Adjustment of the pH Upon the Alga Grown at 21° C, and Harvested After 22 Days.

Daily pH adjusted to	Avg. pH	Avg. dry wt. mg/100 ml	Percent of control
no adjustment (C)	9.4	31.4	100.0
9.0	9.2	23.2	73.9
10.0	9.3	9.9	28.9
11.0	-	alga killed after first few adjustments	0.0
12.0	-	alga killed after first adjustment	0.0

Very poor growth resulted in all of those series receiving a pH adjustment (Table 29). This was probably due to toxic concentrations of the acid and base which were necessary to effect the desired adjustments in a buffered medium. This postulation seems reliable as death of the alga occurred immediately after the first adjustment of the 12.0 series.

Table 29

Average pH of the Medium

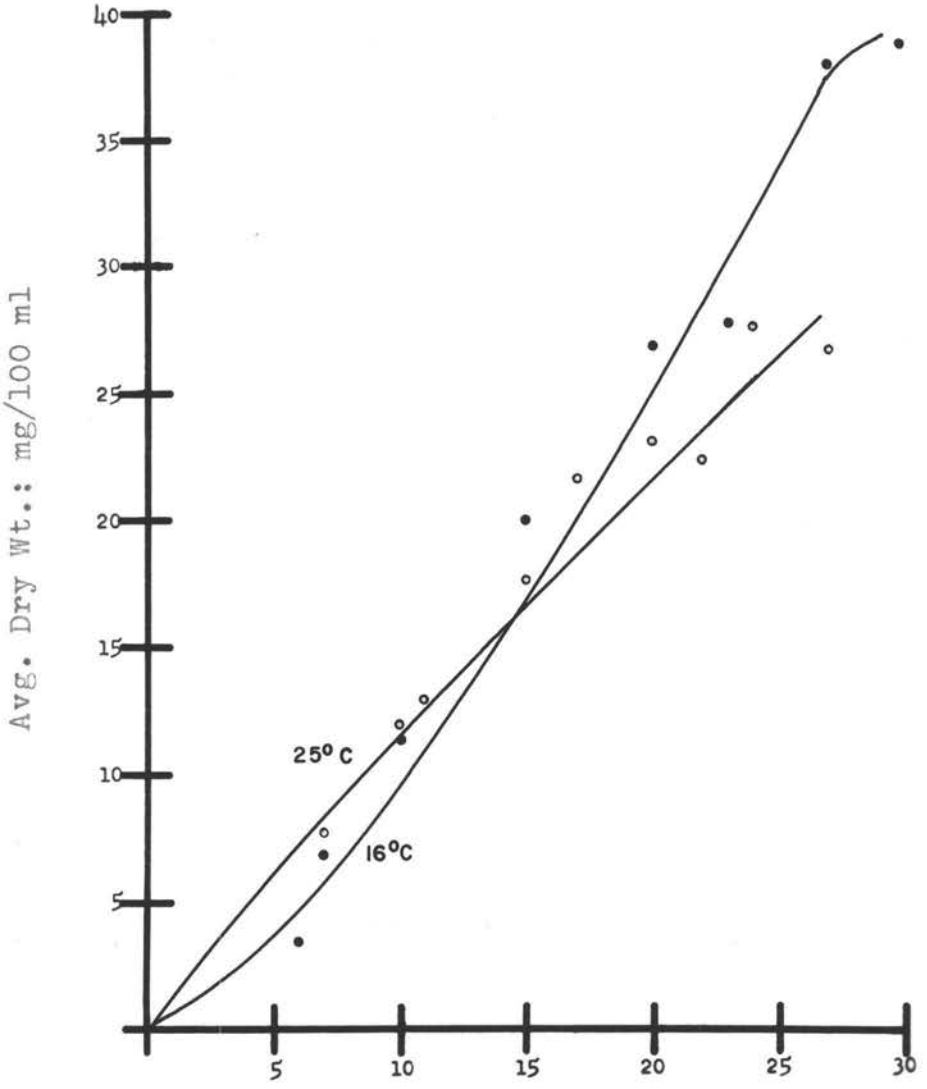
Daily pH adjusted to	Days after inoculation												
	6	7	8	9	10	11	12	13	14	15	16	17	18
9.0	8.1	8.8	8.9	9.1	9.2	9.3	9.4	9.4	9.4	9.6	9.4	9.5	9.5
10.0	8.3	9.5	9.5	9.6	9.8	9.7	9.8	9.8	9.8	9.8	9.8	9.8	9.6
11.0	8.4	9.9	9.9 -- Alga killed after second pH adjustment										
12.0	8.5 -- Alga killed after first pH adjustment												

E. Temperature

Due to the inadequacy of available facilities, it was not possible to make a direct comparison of the growth of 1032 at variable temperatures. The results presented in Figure 4 are an indirect comparison of the growth of the alga at 25° C at Wisconsin in 1955 and at 16° C at Oregon State College in 1956. These data indicated that no detrimental effects were imposed upon the growth of the alga at the lower temperature. These data may also have indicated that the alga grew better at temperatures below 25° C, or during the time between these two experiments, a better growing strain of the alga may have been selected. The greatest degree of difference in these two growth patterns was the large increase in dry weight of the alga after the third week at 16° C. At the lower temperature, maximum growth seemed to be closer to four weeks than to three weeks. At the end of the growth period, the pH of the medium fell to 8.5, indicating a possible cessation of growth. At the higher temperatures, the rate of respiration may have been increased, and a decrease of the accumulated food supply of the alga may have occurred more rapidly. As a consequence, the dry weight of the alga would concomitantly have been decreased. In addition, the greater solubility of carbon dioxide at the lower

Figure 4

Comparison of the Growth of 1032 at 25° C and at 16° C



temperature may have provided a more favorable environment for the growth of the alga. Until a further study of temperature effects can be made, a delay in conclusions seems mandatory.

DISCUSSION

This thesis presents the results of what is believed to be the first successful attempt to grow Aphanizomenon flos-aquae experimentally under controlled cultural conditions.

This alga had previously been suspected of requiring an unknown organic nutrient supplement. This assumption has now been questioned as the alga has been grown in various media containing no organic material other than a citrate iron complex. Even this material was successfully replaced by ethylenediaminetetraacetate, a compound reputed to be metabolically inactive.

The assumption that certain nutrient minerals, particularly nitrogen, phosphorus and iron, are sometimes limiting in nature was supported by the results of this study as far as nitrogen and phosphorus are concerned. The status of iron remains problematical.

A study of the role of the minor elements was entirely omitted. It is proposed that this relationship be explored in the near future, as further knowledge of these nutrients may greatly help elucidate approaches to the quantitative and qualitative control of phytoplankton populations.

The difficulties in evaluating the limiting effect of these nutrients reside in the differences between the cultural system and the natural system. The natural

habitat represents a cyclic system, whereas the cultural habitat represents a "one-way circuit." In addition, the mobility of both planktonts and medium in the natural habitat add to the difficulties of relating water analyses to amounts of minerals actually available to the bios (organisms, planktonts, etc.).

The growth of phytoplanktonts in nature usually proceeds at an exponential rate that may be altered by conditions other than those effected by the algae in staling the medium. The growth of Aphanizomenon in culture typically experienced a lag period immediately following inoculation. This was followed by an exponential stage of growth, after which the growth rate declined, and then leveled out to become constant. The high initial concentration of nutrients may inhibit growth. As these nutrients are absorbed, a more favorable concentration is presented and the exponential phase initiated. A limited carbon supply may cause the lag period. As carbon dioxide slowly diffuses into the medium, the growth rate increases.

A medium ideally contains essential elements in sufficient quantities for luxuriant growth. In some cases, these minerals may be present in large enough quantity to inhibit growth. Nitrogen, as nitrate, and iron, as NaFe-EDTA, in relatively high concentrations apparently inhibited the growth of Aphanizomenon. On the other hand,

the trace element nutrients may be precipitated from an alkaline medium in an unavailable form. This may have been the case with iron. The citrate supplied to stabilize the iron may have been metabolized which would have resulted in a considerable amount of iron being rendered unavailable. This may explain the lack of iron toxicity when large quantities of iron were added as ferric citrate-citric acid.

The preferential absorption of a particular ion may result in a reaction in which the medium becomes inimical to the growth of an organism. In this study, ammonium acted in this manner, resulting in an almost complete suppression of growth. The uptake of a nutrient is not necessarily correlated with the metabolic requirements of the organism. Phosphorus was accumulated in large quantities within the cells of the alga (up to 0.02 ppm may have accumulated in the cells of the inoculum). The accumulated phosphorus supported a small amount of growth in a medium containing no phosphorus. The amount of phosphorus carried over was probably related to the age of the culture used as a source of inoculum; the younger the culture, the more phosphorus accumulated per cell. Nitrogen may also be accumulated, but accumulation generally occurs under conditions of nitrogen deficiency. As the inoculum was always taken from young cultures, no indication of nitrogen accumulation was evident. Iron, too, can

apparently be absorbed in excess of the metabolic demands. This would account for the toxic effects exhibited by the alga when iron was supplied as NaFe-EDTA.

In cultures with limited volume of medium, exponential growth of the organism is sooner or later arrested. The rate of growth may be affected in numerous ways. For example, exhaustion of a nutrient substance from the medium may limit the rate of growth. Each one of the major nutrients has been shown capable of acting in this capacity.

The decline of the growth rate during the third phase may result from a decreasing supply of nutrients or from a reduction in light penetration into the bulk of the medium which would then maintain photosynthesis at a level insufficient to support exponential growth. The former assumption seems more reasonable as the growth rate varied directly with variation of the concentration of nutrients in the basic medium. At the time of harvesting, it was evident that growth was limited in the cultures containing the lowest nutrient content. Growth in the 2X and 4X series exhibited limited growth to a lesser extent. The lag period was longer (by empirical observation) probably as a result of toxic levels of concentration of some ions. The exponential phase was probably limited in the 2X series by the availability of carbon

while in the 4X series the high concentration of nutrient elements may have posed a toxic level throughout the growth period.

In general, the growth rate during the grand period of growth seemed to be related to carbon dioxide diffusion, size of inoculum, and concentration of nutrients present.

The planktonts in nature tenant a much more dilute medium than that contained within the cultural flask. The volume of medium available to the planktonts in nature is, however, much larger. As these populations are able to move throughout the medium, and as the medium undergoes mixing, these organisms actually come into contact with a much larger volume of medium and thereby a considerable quantity of nutrients.

The results obtained indicated that the concentrations of nitrogen, phosphorus, and possibly iron present in natural waters are most likely to exert a limiting effect upon the growth of the alga. The concentration of the two former elements in nature almost invariably falls below the level for maximum growth as determined in these studies. Nitrogen is more likely than phosphorus to be limiting in the growth of the alga. Approximately 40 times more nitrogen than phosphorus was necessary for maximum growth. However, more phosphorus than nitrogen was accumulated.

This may enable the alga to survive better under conditions of phosphorus deficit than under nitrogen deficit.

Nitrogen in relatively high concentration (20.0-25.0 ppm) was shown to exert a tendency to limit growth. These concentrations, however, rarely obtain in nature. Inhibitory concentrations of phosphorus, if such exist, were not determined in the study, but even the concentration necessary for optimum growth is rarely realized in the natural habitat.

Good growth of planktonts in nature in the presence of low concentrations of nitrogen and phosphorus may be explained, at least in part, by the ability of organisms to accumulate these mineral nutrients. If the population is large and growing under a nutrient deficit, these nutrients would probably be taken up as rapidly as they are rendered metabolically available. If these nutrients are absorbed in an organic form, they could function as a nutrient reserve undetected in ordinary inorganic analyses. Such an accumulation of nutrients would lead to water analyses revealing concentrations lower than those actually available for metabolism in the organisms. It would, consequently, be possible for a larger population to develop than would be assumed from water analyses. The problem in establishing iron requirements results from a lack of information concerning those forms of iron which

are available for metabolism and in a lack of acceptable procedures to use in analyzing for these sundry forms.

The experiment which employed Klamath Lake water as the basis for various media presented several interesting aspects. The series to which no nutrients were added resulted in approximately as good growth as the series to which nitrogen, phosphorus, and iron had been added individually. The series which received an enrichment of all three of these nutrients did not result in increased growth. When this water was enriched with nutrients of a complete medium (G-10 or R-VIII), growth exceeded that which had heretofore been achieved in either medium alone. This indicates that nutrients other than nitrogen, phosphorus, and iron were limiting and that this water also contained some substance or substances that stimulated growth. If inorganic, this material was something other than the elements utilized in the basic medium, or there may have been one or more heat stable organic substances present. As the water was collected at a time when no large cyanophycean population was present, the chemical composition of the water may not have been strictly comparable to that obtaining during a blue-green growth period.

Calcium was shown to be necessary in the nutrition of this alga. The calcium ion was required in relatively

small amounts, and in larger quantities it effected an inhibition of growth. The alga in nature is associated with eutrophic habitats, and these habitats are often hard water lakes possessing a high calcium content. No evidence was available which would elucidate the role of calcium in nature as it is related to general phytoplankton development. A possible explanation may reside in the carbonate content of natural waters. The presence of this ion may mitigate the detrimental effects of calcium in a physiologically balanced medium. The inhibition caused by high calcium levels in culture may have a direct effect on the metabolism of the organism, or it may induce some other condition in the medium unfavorable for growth.

It is not surprising that an alkaline medium favored the development of this organism as the alga is associated in nature with waters having a relatively high pH. The optimum pH for the development of Aphanizomenon may be somewhat above that of natural waters in which the alga is usually found. An initial pH of 8.0 did not seem to cause any serious harm to the alga, although at this value growth was considerably retarded in comparison to that which was obtained at higher values. The pH of natural waters is an extremely variable factor, and direct effects of the hydrogen-ion concentration are difficult to evaluate.

As very little work was done in relation to growth as it was affected by light, no conclusions can be made. In nature, as under cultural conditions, the light intensities are relatively low. In the natural habitat, much of the light is reflected from the surface of the water, while another large portion of light is absorbed by the water. In addition, the suspended material and natural color of the water are effective filters and decrease the intensity of the light. The light intensity is also greatly reduced by mutual shading by the planktonts, and to some extent this probably took place in the flasks. Those planktonts lying at the surface of the water may be in a state of senility, so that the effect of higher light intensity is of no consequence, or there may be a rapid turnover of the individuals at the surface which results in no one cell being exposed for any length of time to a high light intensity. The alga in culture seemed to require a low light intensity (ca. 100 f.c.). The intensity of light required, as related to the growth of the alga, was probably in turn related to the conditions under which the alga was grown, and this requirement may be altered as the other cultural conditions are altered.

From the results obtained with this alga by various investigators, it seems probable that within undetermined upper and lower limits, the temperature has very little

effect upon the growth of the alga. The lower temperatures used at Oregon State seemed to favor greater growth. One possible explanation may be that the lower temperatures favor a lower respiratory rate without seriously decreasing the rate of photosynthesis. This would tend to promote a higher ratio of photosynthesis to respiration. Consequently, the weight of the alga would decrease more slowly in an effete medium under lower temperature conditions.

Bacterial contamination in the cultures was believed not to have posed a serious problem by reasons of the following considerations: (1) The inorganic nature of the medium would tend to discourage the multiplication of bacteria. (2) The pH at which most of these experiments were performed would further tend to inhibit the growth of bacteria. (3) If large bacterial populations were present in the cultures, one would expect a breakdown of the citrate complex which would have resulted in a tendency for iron deficiency to appear. (4) The low pH values obtained by using ammonium as a source of nitrogen indicated that ammonia was not converted to nitrate by bacterial action before it was absorbed by the alga. (5) If nitrogen-fixing bacteria were present, the combined nitrogen source in the medium would tend to suppress their activities (72, p.341). (6) The fact that there was an almost complete lack of growth in those cultures to which

no nitrogen had been added also suggests that nitrogen-fixing bacteria were not present.

The results which were obtained using A. flos-aquae were quite similar to the results of Gerloff et al. (31, pp.26-32) working with Anacystis cyanea. The question then arises whether these two organisms possess similar metabolic requirements or whether the results reflect the similarity of cultural conditions imposed upon these two species. Inasmuch as these forms are commonly associated as major components of bloom populations, some merit in the former proposition can be acknowledged.

Strains which were adapted to the specific cultural conditions may have been selected out of original physiologically diverse populations. The applicability of results obtained under cultural conditions are, therefore, equivocal. Relative to this question, several points must be considered. One serious aspect arises from the possible effects of ultra-violet irradiation used in producing bacteria-free cultures. This technique may invalidate the results insofar as the organism in culture represents the species as found in nature. The mutagenic properties of these rays may have induced a change in the population, or part thereof, which was favored by the cultural conditions. As a result, a strain adapted to these cultural conditions may have been selected

out of an original population. If a selection occurred, it may have been one of two types. A mutant may have arisen following the irradiation, or a strain resistant to the deleterious effects of irradiation and favored by the cultural conditions may have been selected.

A second consideration concerns the procedure used in maintaining subcultures. The inoculum for each succeeding subculture was taken during the period of maximum growth. The original cultures produced very poor growth, and the eventual success may have been due to a cumulative selective process, resulting in a strain which was favored by the procedure used. There may also have been a selection of a contaminant, that growing symbiotically with the alga, could possibly account for increased growth. An indication that subculturing selection was in operation was borne out by the early experiments. In these experiments, frequently some of the cultures did not yield as good growth as others within the same series. In addition, during the early stages of this study, many of the experiments had to be discarded, and new ones established as a result of lack of growth. During the course of these experiments, the average dry weight of the control series increased by approximately 11 percent. However, inasmuch as the physical conditions were altered somewhat, the increased

growth may reflect these conditions rather than the alternative assumption. The later experiments were for the most part successful, and only a few had to be repeated because of unfavorable growth. During this time, it would have been unnecessary to carry the usual four flasks in each series as three would have sufficed.

The morphological features of this alga in culture may also indicate that an atypical form was used. Strain 1062, which was used in the early part of this work, had not been subjected to ultra-violet irradiation and grew in typical bundles. This would lead one to adopt an explanation based upon considerations other than cultural conditions.

Previous investigators who were unsuccessful in culturing Aphanizomenon may have worked with different strains that demanded unknown organic metabolites or growth substances. These workers may also have used physiologically unsuitable inocula. If the medium employed and the physical conditions are suited for this alga, one ought to be able to easily introduce it into culture. However, such has not been the case, which suggests that perhaps only a small portion of the population is physiologically adapted to the cultural conditions. If this is true, to attempt to introduce this species into

culture is a seemingly sisyphean task, and persistence is the only hope of establishing cultures of this planktont.

No claims are made here that this thesis presents the complete details of the nutrition of Aphanizomenon flos-aquae. To establish a claim of this scope, one would have to study representatives from the same population, from different populations representing the same biotope, and representatives from different biotopes. Vindication of this work resides in the fact that some of the preliminary problems in culturing this alga have been eliminated. These results make it possible to plan research that should lead to a more complete understanding of the metabolism of this species.

SUMMARY

This thesis describes some of the effects of variations in the concentration of major mineral nutrients upon the growth in culture of the blue-green planktont Aphanizomenon flos-aquae Born. et Flah. In addition, some of the effects of hydrogen ion concentration, temperature, light, and various media were considered. The alga was grown in Erlenmeyer flasks under unialgal conditions. An attempt was made to provide constant conditions of temperature and illumination. Growth was determined by the dry weight method, and the alga was harvested approximately three weeks after inoculation. The initial work of this investigation was carried on at the University of Wisconsin, and the remainder of the experiments were prosecuted at Oregon State College.

The alga had been isolated from plankton collections obtained from southeastern Wisconsin. The organism was originally grown in a slightly modified version of the Chu number 10 medium. Later the alga was maintained in a physiologically unbalanced medium developed by G. C. Gerloff during investigations of Anacystis cyanea. The culture as used in this experiment, although unialgal, was not bacteria-free. A number of unsuccessful attempts had been made to render this strain bacteria-free by the

use of ultra-violet irradiation and antibiotics. The alga as grown under culture conditions was morphologically atypical. The bundles of filaments characteristic of the species were not evident, nor were akinetes observed.

All of the major mineral nutrients, including iron, which have been found to be necessary for the growth of higher plants were also found to be necessary for the growth of the alga. Of these nutrients, nitrogen was found to be required in the largest amount, and its requirement greatly exceeded that of phosphorus. Both of these nutrients were demanded in much larger quantities than those usually found in the natural habitat. Iron supplied as ferric citrate-citric acid was required in larger quantities than that normally required for a minor element. Using a chelated form of iron, the concentration needed to satisfy the requirement was considerably reduced. When chelated iron was supplied in the concentrations used with ferric citrate, it was found to be toxic to the growth of the alga. This suggested that a considerable amount of the iron supplied as ferric citrate was rendered unavailable. A definite requirement for calcium was shown, but the concentration necessary for good growth fell considerably below that necessary in the nutrition of higher plants. Strontium was not able to replace calcium in the nutrition of the alga.

Initially, the best growth of the alga occurred in a physiologically unbalanced medium. Using a physiologically balanced medium (Rodhe VIII medium), growth always fell below that obtained in the G-10 medium. The main factor inhibiting growth in the R-VIII medium was apparently the high calcium content. By reducing the calcium concentration and increasing the nitrogen and iron to levels comparable to those found in the G-10 medium, growth in a physiologically balanced medium was as good as that which had been obtained in the physiologically unbalanced medium.

Experiments relating pH to growth were inconclusive. This work did show, however, that an alkaline medium was required for growth of the alga. Sodium carbonate and sodium silicate effect a weakly alkaline reaction, and as nitrate was absorbed, the pH of the medium increased, seemingly favoring growth. No quantitative experiments were carried on relating light or temperature to growth. High light intensities were found to be inimical to growth. Within the temperature ranges used in these experiments, no major differences in growth were noted. If there were any temperature effects, growth of the alga seemed to be favored by the lower temperatures used.

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